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Journal of Embryology and Experimental Morphology

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Radiodestructions partielles chez l'embryon de Poulet aux stades jeunes et localisation des ébauches digestives

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AVEC UNE PLANCHE

La localisation des territoires présomptifs du tractus digestif de l'embryon de Poulet a été établie aux stades initiaux du développement. En particulier, les travaux de Hunt (1932), Dalton (1935), Rawles (1936) et Rudnick & Rawles (1937) ont montré qu'aux stades de la ligne primitive et du prolongement céphalique, ces territoires s'échelonnent le long de la ligne primitive. Au niveau de la première moitié de cette ligne se situe le territoire de l'intestin antérieur et de ses annexes. Le reste du tube digestif se trouve en arrière de cette zone.

Ultérieurement, c'est au stade de 30 somites environ que les premières différenciations de l'intestin primitif deviennent discernables. Mais, pendant la période comprise entre ce stade et la formation des premiers somites, la localisation des différentes parties du tractus digestif n'est pas établie.

La méthode des radiodestructions localisées aux rayons X mise au point par P. Ancel & Ét. Wolff (1934) et qui a été utilisée par Ét. Wolff (1936) en particulier pour localiser les ébauches présomptives des membres, nous a permis d'aborder cette question.

TECHNIQUE

Les embryons ont été irradiés aux stades de 9 à 25 somites selon les séries expérimentales (stades 10 à 15 de Hamburger & Hamilton, 1951).

Nous avons utilisé le tube Machlett type A.E.G. 50–T à anticathode de tungstène de 5 mm.[2] et fenêtre de beryllium de 1 mm. d'épaisseur, sous une tension de 15 KV. et à une intensité de 21 mA. Le localiseur est un tube de plomb de 7 cm. de longueur et 3 mm. de diamètre intérieur. L'embryon est à 13 cm. de l'anticathode. Les régions devant être protégées sont recouvertes d'un écran de plomb de 1 mm. d'épaisseur.

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RÉSULTATS

Irradiation en arrière d'un somite déterminé (durée de l'exposition aux rayons X: 3 minutes)

Pour obtenir des survies dépassant 7 jours, on protège la région toute postérieure de l'embryon, où se formera l'allantoïde. Les limites antérieures et postérieures des irradiations sont représentées sur les figs. 1 et 2.

Les séries expérimentales sont résumées dans le tableau 1. Quelle que soit l'étendue de la zone irradiée, les deux parties protégées se raccordent toujours par un court pédicule, qui ne possède généralement pas de germes plumaires.

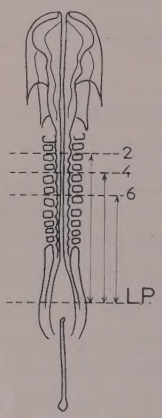

FIG. 1

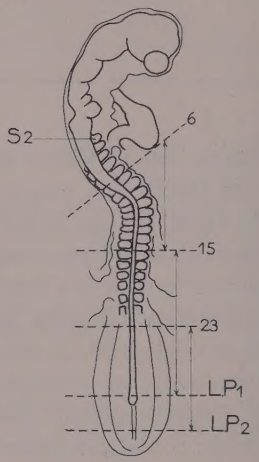

FIG. 2

FIG. 1. Embryon de 11 somites sur lequel sont représentées les limites des irradiations pratiquées en arrière des 2ème, 4ème, 6ème somites. *LP*, limite postérieure des irradiations.

FIG. 2. Embryon de 21 somites sur lequel sont représentées les limites des irradiations pratiquées: entre les 6ème et 15ème somites, en arrière du 15ème somite, en arrière du 23ème somite (on a figuré le niveau approximatif de l'apparition du 23ème somite). S_2, 2ème somite; $L.P._1$, limite postérieure de l'irradiation pratiquée en arrière du 15ème somite; $L.P._2$, limite postérieure de l'irradiation pratiquée en arrière du 23ème somite.

TABLEAU 1

Les séries expérimentales

Irradiation en arrière du	*Stades (nombre de somites)*	*Nombre de cas*	*Survie ⩾ 7 jours*
23ème somite	23 à 25	6	6
15ème somite	19 à 25	44	11
6ème somite	10 à 20	30	7
4ème somite	10 à 18	34	9
2ème somite	9 à 15	112	19

Irradiation en arrière du 23ème somite. Dans ces expériences, la partie postérieure protégée a été réduite. Le tronc est sectionné en avant des membres postérieurs. On n'observe pas de cœlosomie. L'intestin postérieur (c'est-à-dire le rectum et les cæcums) est absent et la grande anse intestinale se termine en cul-de-sac. On remarque donc que la lésion du tube digestif se situe au même niveau transversal que celle du tronc.

Irradiation en arrière du 15ème somite. La section du corps se situe à la base du cou et les ailes sont absentes. La cœlosomie est fréquente comme on peut le voir sur l'embryon représenté figure B de la Planche. La fig. A correspond à un témoin du même stade.

Le tube digestif présente une déficience au niveau de la grande anse intestinale. Celle-ci est en général totalement supprimée. Dans certains cas elle subsiste sous forme d'un fin tractus sans lumière, ou de vésicules à parois minces. La partie postérieure de l'embryon, assez largement protégée, comprend le rectum et les cæcums. Le pédicule vitellin se rattache au rectum.

Irradiation en arrière du 6ème somite. Le cou est incomplet. Les viscères sont logés dans une poche à paroi mince faisant hernie à la base du cou (Planche, fig. D).

Le tube digestif est sectionné au niveau du gésier dont le tiers postérieur environ est supprimé. Le foie est réduit à une masse de parenchyme adhérente à l'extrémité de la partie du gésier qui subsiste. Le pancréas est absent. La portion caudale de l'embryon, respectée par l'irradiation, comprend le rectum et les cæcums.

Irradiation en arrière du 4ème somite. Le cou est très court. La coupure du tube digestif se situe dans la partie antérieure du gésier. Le foie n'est représenté que par une quantité très réduite de tissu collé à l'extrémité du tube digestif.

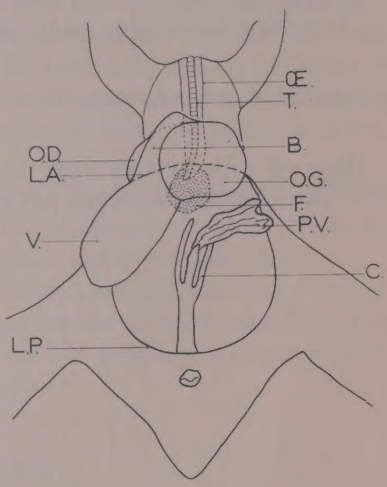

FIG. 3. Vue ventrale d'un embryon irradié en arrière du 2ème somite. Cet embryon présente une cœlosomie dont la limite antérieure (*L.A.*) se situait environ au milieu du cœur. Le tube digestif est interrompu en arrière de l'œsophage et en avant du rectum. *B.*, bulbe aortique; *C.*, cæcums; *F.*, foie; *O.D.–O.G.*, oreillette droite – oreillette gauche; *Œ.*, œsophage; *P.V.*, pédicule vitellin; *T.*, trachée; *V.*, ventricule; *L.A.–L.P.*, limites antérieure et postérieure de la cœlosomie.

Irradiation en arrière du 2ème somite. Le cou est absent. Dans ces expériences, la partie postérieure protégée a été importante (Planche, fig. C).

Dans 10 cas sur 19 on observe une cœlosomie. Celle-ci peut intéresser la moitié postérieure des ventricules, qui présentent une constriction médiane. Dans d'autres cas, la cœlosomie comprend le cœur entier. Le péricarde ventral

est alors soudé à la paroi du cou, inversant ainsi la position de l'organe dont les ventricules sont dirigés crânialement (Planche, fig. C).

La section du tube digestif se situe en avant du ventricule succenturié qui est absent. Un très petit fragment de tissu hépatique adhère à l'extrémité de l'œsophage, et cet ensemble entre en connection avec la paroi dorsale du sinus veineux. Le reste de l'appareil digestif est détruit, à l'exception de l'intestin postérieur qui se trouve dans la région postérieure protégée (fig. 3). La trachée-artère est sectionnée avant la naissance des bronches qui sont supprimées ainsi que les poumons, tandis que ces organes ont toujours été respectés par les irradiations précédentes.

Le cœur est le plus souvent anormal: le bulbe artériel n'effectue pas la rotation qui le ramène sur la ligne médio-ventrale.

Irradiation d'une zone transversale étroite (durée de l'exposition aux rayons X: 4 minutes)

Dix-neuf embryons au stade de 20 somites ont été irradiés entre le 6ème et le 15ème somites. Dix d'entre eux ont survécu plus de 7 jours et ont pu être disséqués.

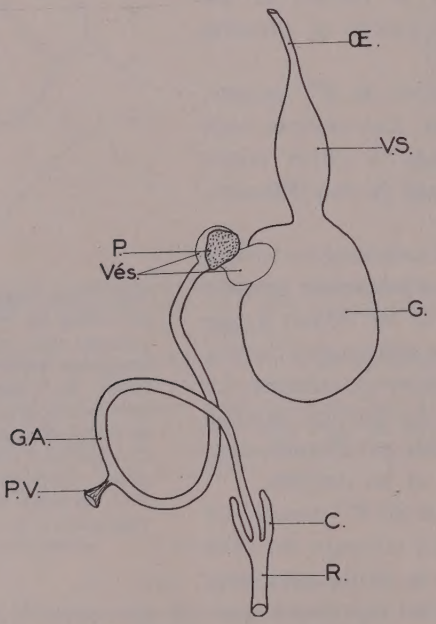

FIG. 4. Tube digestif d'un embryon irradié entre les 6ème et 15ème somites. L'anse duodénale courte est formée de vésicules à parois minces (*Vés.*). Le pancréas est de taille réduite et de forme anormale, mais le tube digestif est continu. *C.*, cæcums; *G.*, gésier; *G.A.*, grande anse intestinale; *Œ.*, œsophage; *P.*, pancréas; *P.V.*, pédicule vitellin; *R.*, rectum; *V.S.*, ventricule succenturié.

Ils présentaient une constriction au niveau du cou. Le tronc et les membres sont normaux.

Du point de vue de l'organisation interne, les résultats peuvent se classer en deux catégories: (*a*) absence de lésion apparente des organes internes (3 cas), et (*b*) tube digestif continu mais présentant des lésions (7 cas). La partie distale du gésier est de forme anormale. L'anse duodénale est courte et comporte fréquemment des vésicules à paroi mince. Le pancréas est de volume réduit (fig. 4).

Donc la lésion des organes axiaux conditionnant la morphologie externe se trouve au niveau du cou, tandis que celle du tube digestif se situe dans le tronc. D'autre part, la lésion est moins importante dans le territoire correspondant à l'irradiation entre le 6ème et le 15ème somites que dans ce même territoire lorsqu'on irradie du 6ème somite à la région terminale. Et cependant, la dose de rayons X administrée a été plus faible dans ce dernier cas. Il apparaît donc qu'une certaine réparation des lésions est possible lorsque le champ de l'irradiation est réduit.

Irradiation unilatérale droite (*durée de l'exposition aux rayons X: 4 minutes*)

Elle a été pratiquée entre le 6ème somite et la région tout à fait terminale de l'embryon et intéresse les somites et les plaques latérales du côté droit.

Dix embryons au stade de 15 somites ont été irradiés. Six d'entre eux ont pu être étudiés: du côté droit, la paroi du tronc et les membres manquent. L'intestin est continu, mais de calibre nettement inférieur à la normale. Le cæcum droit manque. Le lobe droit du foie est de taille réduite.

CONCLUSIONS

1. Les expériences de radiosections transversales permettent de localiser les ébauches des différentes parties du tractus digestif, ainsi que les poumons, aux stades considérés (9 à 25 somites) (fig. 5).

En avant du 2ème somite: œsophage.

Entre le 2ème et le 4ème somites: ventricule succenturié, partie antérieure du gésier, poumons.

Entre le 4ème et le 6ème somites: partie moyenne du gésier.

Entre le 6ème et le 15ème somites: partie terminale du gésier (la limite postérieure de ce dernier semble se situer en arrière du 7e somite), anse duodénale et pancréas.

Entre le 15ème et le 23ème somites: grande anse intestinale.

En arrière du 23ème somite: intestin postérieur.

Les expériences réalisées n'ont jamais supprimé totalement le foie. Le territoire présomptif de cet organe dépasse donc en avant le 2ème somite. De même, sa limite postérieure n'a pas été établie avec précision. Elle dépasse en arrière le 6ème somite.

2. Les séries expérimentales étudiées montrent que pour une irradiation

postérieure (en arrière du 23ème somite) la lésion des organes axiaux et celle du tube digestif se situent au même niveau transversal. Par contre, les irradiations antérieures produisent un décalage entre les deux types de lésion. Tandis que l'ectoderme et le mésoderme situés en avant du 15ème somite appartiennent au cou, l'endoderme correspondant fournit non seulement l'œsophage et le jabot, mais aussi des organes abdominaux: l'estomac et l'anse duodénale.

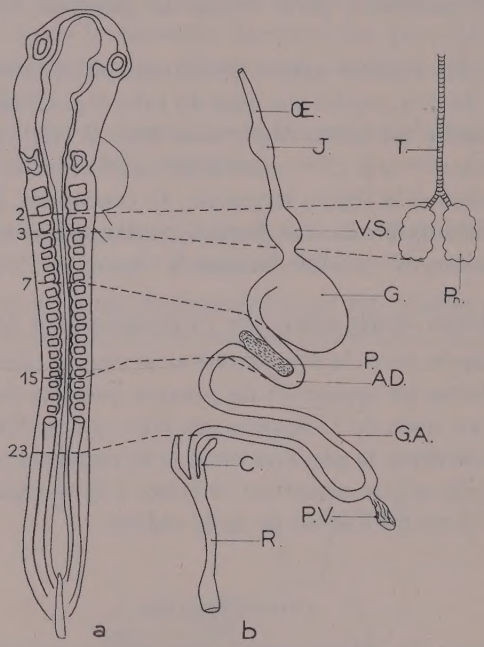

FIG. 5. Localisation des ébauches digestives. La fig. 5 représente sur un embryon de 18 somites (fig. 5*a*) l'emplacement des ébauches des différentes parties du tube digestif et de l'appareil respiratoire schématisés fig. 5*b*. *A.D.*, anse duodénale; *C.*, cæcums; *G.*, gésier; *G.A.*, grande anse intestinale; *J.*, jabot; *Œ.*, œsophage; *P.*, pancréas; *Pn.*, poumon; *P.V.*, pédicule vitellin; *R.*, rectum; *T.*, trachée; *V.S.*, ventricule succenturié.

3. La lésion par les rayons X d'une étroite zone transversale de l'ébauche digestive peut donner lieu à des phénomènes de régulation, la partie irradiée subsiste alors en présentant une morphologie plus ou moins proche de la normale. De même, les irradiations unilatérales montrent que la bande endodermique respectée est suffisante pour constituer un tube digestif, la perte de substance ne se manifestant que par la réduction du diamètre de l'intestin.

RÉSUMÉ

1. Des expériences de radiodestructions aux rayons X ont été effectuées sur

l'embryon de Poulet aux stades de 9 à 25 somites. Les embryons ayant survécu au delà du 7ème jour de l'incubation ont été étudiés.

2. Des radiosections transversales ont été pratiquées en arrière d'un somite déterminé (23ème, 15ème, 6ème, 4ème, 2ème) (figs. 1, 2). Dans ces expériences, la région postérieure de l'embryon a été protégée pour que l'allantoïde puisse se développer. Les deux parties de l'embryon respectées par l'irradiation se raccordent toujours par un court pédicule. On observe parfois une cœlosomie (Planche, figs. B, C). Chez les embryons irradiés en arrière du 6ème ou du 4ème somite les organes sont souvent logés dans une poche située à la base du cou (fig. D).

La destruction des organes internes dans la zone irradiée a permis de localiser aux stades considérés les ébauches des différentes parties du tractus digestif et de l'appareil respiratoire (fig. 5).

3. L'irradiation d'une zone transversale étroite (entre le 6ème et le 15ème somites) fournit des embryons dont la partie du tube digestif correspondant à l'irradiation présente des anomalies mais n'est pas supprimée (fig. 4). De même, des embryons ayant subi des irradiations unilatérales présentent un intestin continu, mais de diamètre réduit. Cependant la dose de rayonnement est supérieure à celle utilisée dans les expériences précédentes. Donc, une certaine réparation des lésions est possible lorsque la zone irradiée est restreinte.

SUMMARY

1. Experimental destruction by X-irradiation of regions of chick embryos (stages 9–25 somites) was carried out to study the formation and development of certain organ rudiments. Only embryos surviving 7 days of incubation were studied.

2. Transverse regions of the embryo localized behind a given somite (23rd, 15th, 6th, 4th, 2nd) were irradiated (figs. 1, 2). The posterior region of the irradiated embryo was shielded to allow normal development of the allantois.

The shielded anterior and posterior portions of the embryo always became connected by a pedicule of tissue; and sometimes a coelosomic condition resulted (Plate, figs. B, C). If the embryos were irradiated behind the 4th or 6th somite, some internal organs were often lodged in a pocket situated at the base of the neck (fig. D).

The destruction of these specific regions has permitted localization of rudiments of different parts of the digestive tract and respiratory system (fig. 5).

3. The second series of experiments utilized a higher dose of irradiation than the preceding series. The irradiation of a narrow transverse zone (between the 6th and the 15th somites) produced embryos in which the part of the digestive tract corresponding to the irradiated region was present but anomalous (fig. 4). Also, embryos subjected to unilateral irradiation possessed a completely continuous intestine with a reduced diameter. Thus some repair of X-ray lesions is possible when the irradiated zone is limited in extent.

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EXPLICATION DE LA PLANCHE

FIG. A. Embryon témoin de 8 jours d'incubation (stade 35 de Hamburger & Hamilton).

FIG. B. Embryon irradié en arrière du 15ème somite. La section du corps se situe à la base du cou. Cœlosomie.

FIG. C. Embryon irradié en arrière du 2ème somite. Absence de cou, cœlosomie. Les ventricules sont dirigés crânialement, la paroi du cœur est anormale.

FIG. D. Embryon irradié en arrière du 6ème somite. Le cou est réduit. Organes logés dans une poche à paroi mince faisant hernie à la base du cou.

F, foie; *G*, gésier; *P*, patte; *Pd*, poumon droit; *Po*, poche contenant les viscères; *V*, ventricules; *O*, oreillette.

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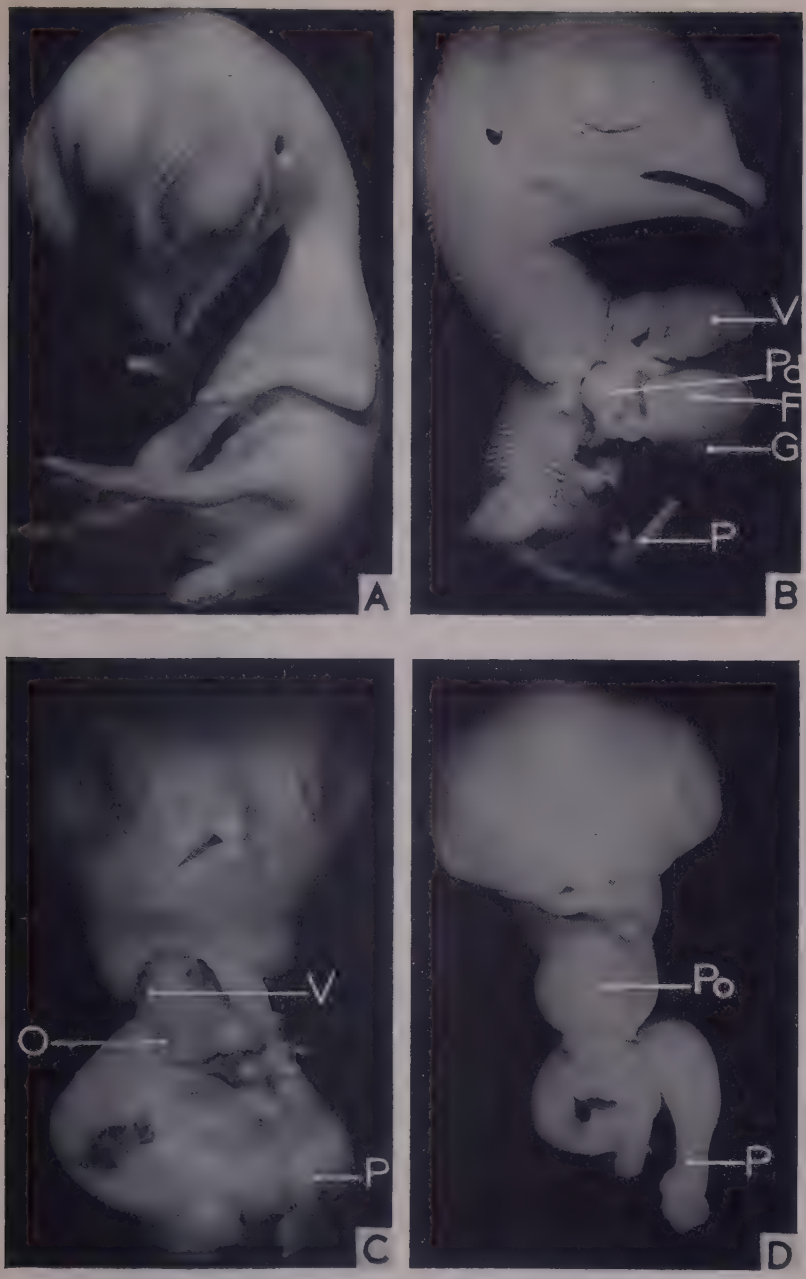
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L'Action de l'irradiation ultraviolette sur le métabolisme des acides nucléiques dans l'embryon de Poulet

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INTRODUCTION

DAVIS (1944) a montré que des doses faibles de lumière U.V. empêchent la fermeture de la gouttière neurale dans l'embryon de poulet.

Se basant sur la région du spectre la plus efficace, cet auteur avait conclu que cette inhibition de la morphogénèse serait la conséquence d'une altération du métabolisme des stéroïdes. Nous avons répété cette expérience afin de préciser si ce traitement n'affecterait pas aussi le métabolisme des acides nucléiques. Nous avons étudié dans ce but, l'incorporation de l'adénine marquée par la méthode autoradiographique.

MATÉRIEL ET MÉTHODES

Six embryons de poulet ayant de 1 à 7 somites ont été dépourvus de leurs membranes vitellines, explantés et cultivés *in vitro* dans le 'minimum medium' de Spratt (1948). Après leur étalement dans le milieu de culture, les embryons ont été soumis à l'irradiation d'une lampe 'Mineralight' pourvue d'un filtre SL 2537, à des doses d'environ 200 erg mm.$^{-2}$ (déterminées au photomètre de Latarjet).

Les embryons ont ensuite été placés dans le milieu de Spratt contenant environ 0·3 μC/c.c. d'adénine-8-C^{14} pendant 2 à 3 heures avant d'être fixés au Serra, coupés et autoradiographiés selon la méthode de Ficq (1955) qui a déjà été appliquée à ce matériel par Tencer (1956) et Barbieri (1960). Dans certaines des expériences, les embryons n'ont été placés dans le milieu radioactif qu'une heure apres l'irradiation. Les coupes destinées à l'autoradiographie ont été colorées au mélange vert de méthyle-pyronine de Unna.

Certaines des coupes ont été soumises à l'action de la ribonucléase (Armour Laboratories; 0·5 mg. c.c., 2 heures à 37° C.), de l'eau distillée dans les mêmes conditions ou de l'acide chlorhydrique normal (5 minutes à 60° C.); cela nous a permis de suivre l'incorporation du précurseur dans l'ADN, l'ARN et le

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résidu résultant de l'hydrolyse des coupes dans HCL N à 60° C., ainsi que dans une fraction soluble à l'eau à 37° C.

L'incorporation du précurseur a été mesurée en comptant le nombre de traces dans l'émulsion photographique; les chiffres obtenus ont été ramenés à une surface arbitrairement choisie de 441 μ^2. Quatre embryons témoins ont été soumis aux mêmes traitements excepté l'irradiation ultraviolette. Nous avons comparé des embryons de différents âges bien que les différences d'activité qu'on peut attribuer au traitement soient beaucoup plus grandes que celles dues à l'âge embryonnaire, comme le montrent les données de Tencer (1956) et les chiffres fournis par nos propres témoins.

RÉSULTATS

Comme Davis (1944), nous avons pu constater une inhibition de la fermeture de la gouttière neurale par l'action des U.V.

Effets sur l'incorporation de l'adénine-C^{14}

(*a*) Immédiatement après l'irradiation, c'est-à-dire au cours de la 1ère et de la 2ème heure, il y a une *augmentation* de l'incorporation totale de l'adénine au niveau des trois portions, *A*, *B* et *C*, du tube neural (*A* étant la portion céphalique et *C* la portion caudale) (tableau 1).

TABLEAU 1

Incorporation de l'adénine-C^{14} (O·3 μC c.c.) dans le tube neural

Expérience	Somites	A		B		C	
		T	*U.V.*	*T*	*U.V.*	*T*	*U.V.*
1. Adénine:							
13 γ/c.c., incub. 1 h.	3	2·3	3·6	4·0	6·3	6·8	8·2
2. Adénine:							
13 γ/c.c., incub. 1 h.	5	0·7	1·7	3·5	9·0	6·8	13·8
3. Adénine:							
6 γ/c.c., incub. 2 h.	7	1·8	—	3·9	—	7·8	—
	4	—	7·0	—	8·0	—	14·5
	5	—	6·5	—	10·3	—	10·1
4. Adénine:							
6 γ/c.c., incub. 3 h.	4	2·5	—	15·5	—	14·5	—
(Début: 1 h. après l'irradiation)	7	—	2·1	—	3·8	—	9·5
	6	—	1·5	—	7·7	—	5·6

T = témoins non irradiés; *U.V.* = 200 ergs/mm.²

Les résultats sont exprimés en nombre de traces par 441 μ^2 pour les niveaux *A*, *B*, *C* du système nerveux (*A* étant la portion céphalique et *C* la portion caudale).

Si on essaie de préciser ce qui se passe dans les diverses fractions biochimiques que l'on peut mettre en évidence après hydrolyse enzymatique ou acide des coupes, nos observations préliminaires permettent d'affirmer que cette augmentation de l'incorporation au cours de la première heure intéresse principalement la fraction hydrosoluble, la fraction résiduelle et l'ARN; au contraire,

l'étude de la fraction ADN ne paraît pas permettre de conclusions bien définies, bien que, en général, l'incorporation semble y être diminuée.

(*b*) Si, après l'irradiation, on attend une heure à 37° C. avant de traiter par l'adénine-C^{14} pendant 3 heures, on observe que l'incorporation de l'adénine dans les cellules neurales diminue considérablement après irradiation; les extractions diverses auxquelles sont soumises les coupes avant l'autoradiographie indiquent que l'ADN et la fraction hydrosoluble sont principalement affectés.

(*c*) Notons qu'en aucun cas nous n'avons observé de variation dans l'affinité des cellules pour le vert de méthyle ou pour la pyronine.

Effets sur la division cellulaire

L'index mitotique des tubes neuraux normaux et celui des embryons irradiés a été déterminé: l'irradiation U.V. entraîne une nette augmentation du nombre des figures mitotiques aux divers niveaux du tube neural, comme l'indique le tableau 2.

TABLEAU 2

Nombre de mitoses pour cent cellules dans le tube neural normal (T) et irradié (U.V.)

Age (nombre de somites)	*Niveau du tube neural*					
	A		*B*		*C*	
	T	*U.V.*	*T*	*U.V.*	*T*	*U.V.*
3	9·6 (41 426)	23·7 (104 438)	7·5 (28 372)	7·2 (26 361)	6·8 (23 338)	8·3 (30 361)
4	2·3 (12 522)	16·2 (69 425)	3·7 (15 405)	12·1 (36 297)	2·2 (10 454)	9·1 (34 373)
5	8·8 (40,453)	13·6 (38 279)	3·5 (14 402)	8·5 (33 152)	1·9 (7 368)	6·2 (21 335)
	—	12·3 (51 414)	—	12·3 (44 358)	—	12·1 (28 231)
–	8·3 (48 578)	18·7 (88 470)	6·3 (26 412)	19·5 (63 322)	7·9 (19 240)	17·9 (81 460)

On a compté, sur neuf embryons, tous les noyaux correspondants d'une coupe pour chaque section (*A*, *B*, *C*, *A* étant la portion céphalique et *C* la portion caudale). (Nombre de mitoses/Nombre de noyaux comptés.)

DISCUSSION

Quatre points semblent ressortir du présent travail:

(1) L'incorporation de l'adénine dans les cellules du tube neural augmente *immédiatement* après l'irradiation U.V., puis elle diminue. Cette augmentation initiale semble concerner l'ARN, le résidu de l'hydrolyse acide et une fraction hydrosoluble; la diminution d'incorporation plus tardive semble surtout intéresser la fraction hydrosoluble.

(2) En ce qui concerne l'incorporation de l'adénine dans l'ADN, elle semble

en règle générale être *diminuée* après l'irradiation U.V.; ce résultat mériterait d'être précisé.

(3) Ces variations du métabolisme de l'*adénine* s'accompagnent d'une augmentation du nombre des figures mitotiques.

(4) Comme l'avait déjà montré Davis (1944), la fermeture de la gouttière neurale est inhibée par l'irradiation U.V.

Il est difficile, à l'aide de ces données, d'établir une corrélation entre ces divers résultats. D'une part, les index mitotiques des segments *A*, *B* et *C* du tube neural sont du même ordre de grandeur; d'autre part, il existe un gradient d'incorporation de l'adénine qui va en augmentant vers la région caudale (Barbieri, 1960).

L'arrêt ou la diminution de l'incorporation dans l'ADN qui s'observe dans la plupart des expériences qui ne dépassent pas 3 heures d'incubation après l'irradiation, peut expliquer la *diminution* de l'index mitotique observée par Davis, 30 heures après l'irradiation. Dans l'expérience présente, on observe cependant une *augmentation* de l'index mitotique (cellules en métaphase ou parfois en anaphase) dans les embryons fixés 1, 2 ou 3 heures après l'irradiation. Ce résultat, qui porte sur l'observation de près de 10.000 cellules, n'est pas nécessairement en contradiction avec ceux de Davis, puisque ces observations ont été faites après un délai beaucoup plus long.

Il est probable qu'il s'agit réellement d'une augmentation du nombre de cellules qui *entrent* en mitose: en effet, dans la plupart des observations portant sur les types cellulaires les plus variés, il a été montré que, pour des doses de l'ordre de celles que nous avons utilisées, l'irradiation retarde l'entrée en mitose avant la prophase; au cours de la mitose elle-même, il est beaucoup plus difficile d'arrêter le processus (Gaulden, 1956). Il est très probable que si nous avions fixé les embryons 30 heures après l'irradiation, nous aurions effectivement observé une chute de l'index mitotique.

L'explication de l'augmentation de la synthèse de l'ARN et du nombre des mitoses dans les heures qui suivent l'irradiation doit logiquement trouver son explication dans un déplacement d'un équilibre dynamique cellulaire, résultant de l'arrêt d'un type particulier de métabolisme au profit de celui de l'ARN; ce dernier est peut-être nécessaire pour déclencher la mitose. Le ralentissement de la synthèse de l'ADN pourrait conduire à une augmentation des précurseurs ou des réserves d'énergie permettant un accroissement de la synthèse d'ARN et des mitoses. Il faudrait cependant savoir comment évolue le métabolisme des autres parties de l'embryon et préciser s'il y a une diminution des mouvements cellulaires, qui rendrait également disponible une certaine quantité d'énergie, avant de tirer des conclusions. Il est certain, en tous cas, qu'on observe effectivement un arrêt des mouvements des replis neuraux. En ce qui concerne le spectre d'action de Davis qu'il a interprété en 1944 comme reflétant une absorption par les stérols, il nous semble plus vraisemblable, à la lumière de nos connaissances actuelles sur le rôle des acides nucléiques et des protéines

dans la morphogénèse (Brachet, 1957), d'attribuer les maxima d'efficacité à 260 mμ et à 285 mμ à l'action des U.V. sur respectivement les acides nucléiques et les protéines.

SUMMARY

1. The incorporation of labelled adenine in the neural plate of ultra-violet irradiated chick embryos has been studied.

2. It has been confirmed that small doses of radiation inhibit the closure of the neural folds.

3. The incorporation of adenine increases immediately after radiation and decreases one hour after the treatment.

4. There is a significant increase of the mitotic index during this first hour.

5. In the light of previous results it is possible to attribute the inhibition of closure of the neural folds to some alteration in the metabolism of nucleic acids or proteins.

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Teratogenic Activity of Several Closely Related Disazo Dyes on the Developing Chick Embryo

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THEt eratogenic activity of several disazo dyes during rat embryogenesis has been investigated by Wilson (1955). In addition to the marked teratogenicity of trypan blue Wilson also demonstrated that Evans blue was moderately active and that Niagara blue 4B and Niagara sky blue 6B were possibly active. On the other hand, several other closely related disazo dyes such as Congo red and azo blue were found to cause no malformations in the surviving offspring.

Since trypan blue has been shown to be teratogenic to the chick embryo (Beaudoin & Wilson, 1958), it is of interest to determine whether other disazo dyes that have been tested in the rat affect the chick embryo in the same or a similar manner.

MATERIALS AND METHODS

Fertile eggs of the White Leghorn chicken were obtained from a commercial hatchery. The eggs were injected either into the yolk sac or subgerminal cavity at 36 hours' incubation (Hamburger–Hamilton stages 9–11). Fresh 0·1 per cent. saline solutions of the dyes were prepared just prior to injection. Eggs injected into the subgerminal cavity received 0·05 ml. of dye solution and eggs injected into the yolk sac received 0·1 ml. (for a description of injection techniques, see Beaudoin & Wilson, 1958). Control eggs were either injected with saline or incubated without injection. The dyes selected for study were trypan blue, Evans blue, Congo red, azo blue, Niagara blue 2B, and Niagara blue 4B (dyes obtained from Matheson, Coleman, & Bell Co., Norwood, Ohio). In all experiments embryos were recovered on the 10th day of incubation, fixed, weighed, and examined for gross malformations. Control eggs were run with each experimental group and handled in an identical manner. Another series of eggs was injected with trypan blue alone at different times during incubation from 0 to 96 hours to test for the span of time through which trypan blue is effective.

RESULTS

Table 1 summarizes the results of the action of several disazo dyes on the embryonic chick when injected either into the yolk sac or subgerminal cavity.

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When the dyes were injected subgerminally trypan blue and Evans blue were found to be teratogenic while azo blue, Niagara blue 2B, Niagara blue 4B, and Congo red were not significantly more teratogenic to chick development than was the injection of saline. On the other hand, when the dyes were injected into

TABLE 1

Teratogenic activity of several disazo dyes on chick development when injected at the 36th hour of incubation

Dye	Total treated		Percentage mortality		Percentage malformed survivors			
	Sub-germinal	Yolk sac	Sub-germinal	Yolk sac	Sub-germinal	P values*	Yolk sac	P values
Trypan blue	101	94	45·5	56·5	72·8	< 0·001	56·2	< 0·001
Evans blue	78	90	44·8	14·4	51·3	< 0·001	5·2	0·52
Niagara blue 4B	94	93	69·3	29·9	34·5	0·10	33·4	< 0·001
Niagara blue 2B	67	86	23·9	9·3	27·4	0·47	0	—
Azo blue	82	93	18·3	10·7	20·9	0·90	0	—
Congo red	95	90	59·0	3·3	35·9	0·08	3·4	0·98
Saline controls	220	141	20·9	12·0	20·1	—	2·4	—
Untreated controls	—	134	—	11·9	—	—	3·4	—

* *P* values derived from X^2 test for independence.

the yolk sac the results differed somewhat. Trypan blue was still markedly teratogenic, but Evans blue was not. All other dyes remained non-teratogenic except Niagara blue 4B which when injected into the yolk sac was a teratogen. The reason for this change in behavior of Evans blue and Niagara blue 4B, depending on their route of injection, is not known.

TABLE 2

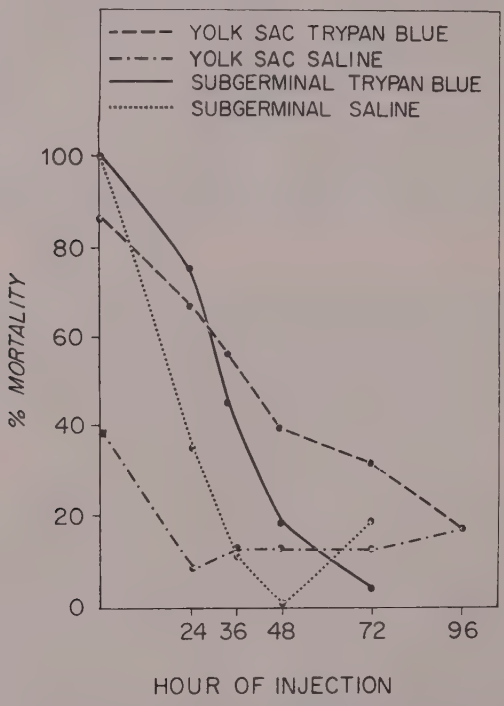
Frequency of malformations among surviving 10-day chicks (percentage of total survivors)

Dye	Rumpless		Eye		Beak		Gastroschisis		Hind limb		Spina bifida		Other	
	SG*	YS†	SG	YS	SG	YS	SG	YS	SG	YS	SG	YS	SG	YS
Trypan blue	69·1	53·6	7·3	2·7	10·9	—	3·6	—	7·3	4·8	—	—	—	2·4
Evans blue	51·2	2·6	7·0	1·3	2·3	—	4·7	—	9·3	1·3	4·7	—	—	6·5
Niagara blue 4B	20·6	31·8	17·2	3·4	17·2	—	3·4	1·5	3·4	—	—	—	—	4·5
Niagara blue 2B	19·6	—	1·9	—	—	—	—	—	—	—	3·9	—	3·9	—
Azo blue	14·9	—	10·4	—	7·5	—	1·5	—	1·5	—	1·5	—	1·5	—
Congo red	33·4	2·3	12·8	2·3	7·7	—	2·6	—	2·6	—	7·7	—	—	—
Saline controls	9·6	2·4	4·6	0·8	1·5	0·8	4·1	—	1·5	—	8·6	0·8	2·5	—
Untreated controls	—	2·5	—	—	—	0·8	—	0·8	—	—	—	—	—	—

* SG = subgerminal. † YS = yolk sac.

Some general statements can be made regarding the types of malformation observed following treatment with the disazo dyes (Table 2). Rumplessness was the most common single defect observed following treatment with dyes or

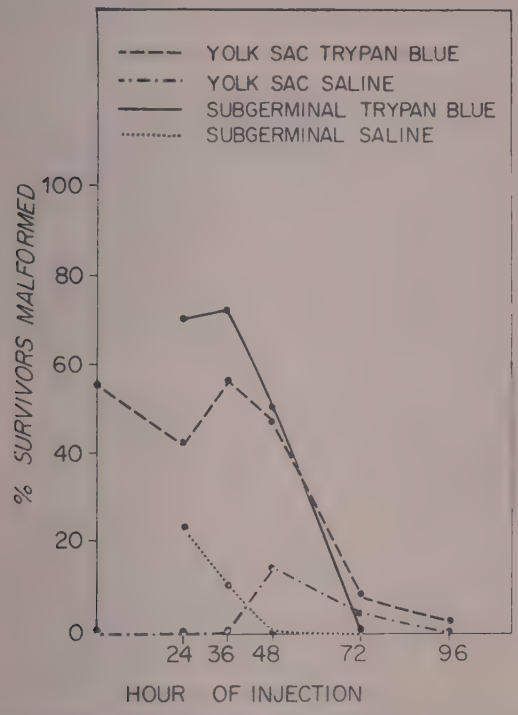
saline, irrespective of the route of injection, and it is noteworthy that rumplessness was also the most common defect among the untreated controls. Two types of eye defects were observed, apparent anophthalmia and various degrees of microphthalmia. Beak defects included cross-beak and deficient beak development. The various other defects were scattered in their occurrence, except spina bifida, which was most common after subgerminal injection, especially following treatment with Congo red and saline. Included in the column headed 'Other' of Table 2 are isolated cases of ectopia cordis, anencephaly, exencephaly, and umbilical hernia. Yolk-sac injections were less traumatic to embryogenesis than injections into the subgerminal cavity. It was found that fewer deaths and abnormalities occurred in embryos from eggs injected into the yolk sac than from eggs injected subgerminally, regardless of the material injected.



TEXT-FIG. 1. The mortality of chick embryos caused by the injection of trypan blue or saline into the yolk sac or subgerminal cavity.

An analysis was made of the duration of effect of trypan blue by injecting this dye at selected intervals during incubation. Text-figs. 1, 2, and 3 show the results of this study. The curves for mortality and numbers of malformed survivors tend to parallel one another. It can be concluded from these figures that trypan blue is an effective teratogen until the 48th or 72nd hour of incubation, after which time little or no teratogenic or lethal effect was seen. If in

addition to expressing the results as the percentage of malformed survivors they are expressed as the percentage of all treated malformed eggs (Text-fig. 3), it is possible to separate the effect of the high mortality from teratogenic activity during the earlier hours of incubation. When this is done it is seen that a peak in susceptibility to the teratogenicity of trypan blue occurred around 36 hours.

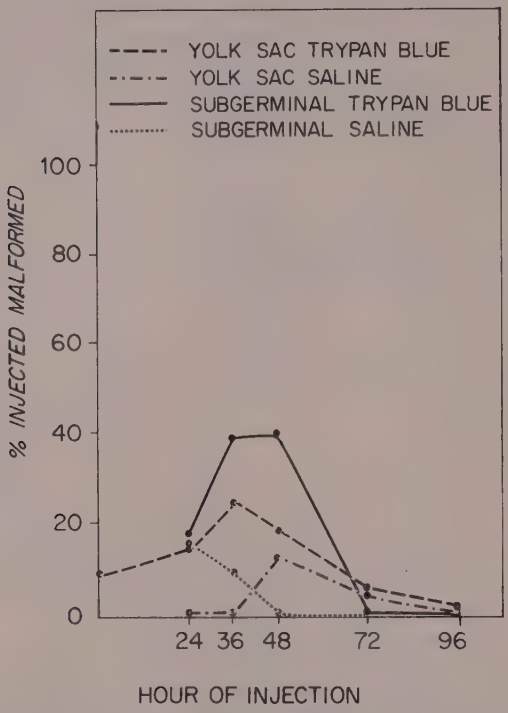


TEXT-FIG. 2. The percentage of 10-day chick embryo survivors malformed following injection of trypan blue or saline into the yolk sac or subgerminal cavity.

DISCUSSION

The results presented in Table 1 for the most part bear out expectations from the work in the rat, in that not all disazo dyes are teratogens. Regardless of the route of injection, trypan blue was markedly teratogenic to the developing chick embryo. However, the teratogenicity of Evans blue and Niagara blue 4B appears to depend upon the route of injection. The remaining dyes tested had no more effect on chick development than did saline. There was always a higher mortality and a higher incidence of malformation among survivors in the subgerminally injected group. This probably is due in part to the mechanical disturbance caused by fluids being forced into the subgerminal cavity under pressure. Landauer & Baumann (1943) have suggested mechanical injury to the blastoderm as a probable cause of rumplessness in chicks when eggs were subjected

to mechanical shaking. In the present study bits of yolk were seen to dislodge and swirl around in the subgerminal cavity when the injection was more forceful than intended. Because of this indication of mechanical disturbance in the original experiment and because of the high incidence of death and abnormality in the saline control series, it was decided to repeat the experiment, but with yolk-sac injections. The results for the yolk-sac injections (Table 1) support the



TEXT-FIG. 3. The percentage of malformed 10-day chick embryos calculated from the total number of eggs injected.

conclusion that the high lethal and teratogenic effect of saline in the subgerminal series may be due in part to mechanical factors, for a significant drop occurred in the mortality and incidence of malformation when the injection was made into the yolk sac. In fact, there were actually fewer malformations in the saline group than in the untreated controls, indicating saline to have no effect as a teratogen in chick development. Nor did such treatment cause any increase in the number of embryonic deaths over the base level established by the untreated controls.

Another factor to be considered is the distribution of the dye following injection. After a subgerminal injection the dye can be observed filling all or a major portion of the subgerminal cavity, thereby presumably coming into direct

contact with cells of the embryo. This is important assuming that there is a direct action of trypan blue on the cells of the embryo. Within one hour after yolk-sac injection the dye had migrated to a point uppermost in the horizontally placed egg, thereby coming to lie directly under the embryo. Regardless of route of injection the dye was never found in any cells of the embryo proper, although cells in the yolk-sac membrane were found to contain particles of the injected dye. The pattern of rise of dye in the egg was the same as that described by Kropp (1957) and Schlesinger (1958). Schlesinger further stated that the migration of substances through the yolk depended upon the relative density of the yolk and of the substance injected. Trypan blue will migrate only very little when injected into yellow yolk, whereas it shows great mobility when injected into the latebra (white yolk). This is due to differences in viscosity, yellow yolk having been estimated to be some 8 times more viscous than the latebral white yolk. Thus, unless dye is injected into the latebra or near enough to it to disrupt the latebra–yellow yolk interface, there will be no migration. Schlesinger has also shown that with an increase in incubation time there is an increase in the speed of migration owing to a decrease in the viscosity of the latebral yolk.

Analysis of the types of defects found in all groups, including untreated controls, showed rumplessness to be the most common single defect observed (Table 2). It is of interest to note that Landauer (1945) reported rumplessness in 2·6 per cent. of his untreated controls and in 2·7 per cent. of his saline yolk-sac injected eggs. This compares with 2·4 per cent. in the untreated controls and 2·5 per cent. in saline-injected eggs of the present study. Only two cases of defects other than rumplessness occurred in the untreated controls, gastroschisis in one rumpless chick, and in another embryo a poorly formed beak as its only defect. Thus the rumpless condition not only occurs spontaneously in the chicks used in these experiments but is the most prevalent of the spontaneous abnormalities. A comparison of the defects found in the chicks with those found in rats showed that many of the same general type of abnormality were present in both. However, the most common defect present in the rat, hydrocephalus, was not observed in any chick embryo. The most susceptible period for the rat is just before organogenesis begins, whereas it appears that the dye can be injected somewhat later in the chick, since organogenesis is well advanced at 36 hours' incubation.

Trypan blue was found to be an effective teratogen until some time between the 48th and 72nd hour of incubation. At this time a marked decrease in its activity occurred. Because the chick embryo develops in a cleidoic system, dye injected during the first hours of incubation will be present when the embryo passes through later susceptible periods. This accounts for the fact that a high percentage of malformations resulted from treatment at any time during the first 48 hours of incubation (Text-fig. 1). There is, however, a high mortality among eggs injected during the first 24 hours, and when allowance was made for this (Text-fig. 3) it was found that a peak in the incidence of rumplessness

apparently occurred at about 36 hours of incubation. This agrees rather closely with 31 hours as observed by Landauer & Bliss (1946) in insulin-induced rumplessness. All major organogenesis in the chick has begun by the 48th hour of incubation, but it is not until the 51st–56th hour that the tail-bud first appears from the primitive knot. Thus any injection prior to the 48th hour would allow dye to be present at the time of formation of the tail. The reason that the 36th hour is more critical may be that the teratogenic effect is on the 'chemical differentiation' of the tail tissue before the time that any morphogenetic change is noticeable. It is not known why the formation of the tail is so susceptible while at the same time other organs are undergoing much more pronounced changes. It is quite possible that trypan blue has a specific rumpless-inducing effect in the chick.

SUMMARY

1. The teratogenic activity of trypan blue, Evans blue, azo blue, Congo red, and Niagara blue 2B and 4B was investigated in the chick embryo, using subgerminal or yolk-sac injections at various times during early incubation.

2. In addition to trypan blue it was found that Evans blue was teratogenic when injected into the subgerminal cavity but was not when injected into the yolk sac. The converse was true for Niagara blue 4B. The remaining dyes tested were no more teratogenic than saline.

3. Rumplessness was the most common type of defect observed following injections with a teratogenic dye, regardless of the route of injection. The most susceptible period for the production of rumplessness was during the first 48 hours of incubation, with a peak at 36 hours.

RÉSUMÉ

L'activité tératogène de quelques colorants 'diazo' de constitutions chimiques très voisines sur le développement de l'embryon de Poulet

1. L'activité tératogène du bleu trypan, du bleu Evans, de l'azobleu et du bleu Niagara 2B et 4B a été étudiée sur l'embryon de poulet, par la technique des injections dans le sac vitellin ou dans la cavité sous-germinale à différents stades du début de l'incubation.

2. On a trouvé qu'en plus du bleu trypan, le bleu Evans est tératogène quand il est injecté dans la cavité sous-germinale; mais il ne l'est pas quand il est injecté dans le sac vitellin. L'inverse est vrai pour le bleu Niagara 4B. Les autres colorants éprouvés ne sont pas plus tératogènes que les solutions salines.

3. L'absence de croupion est l'anomalie la plus commune que l'on observe après les injections d'un colorant tératogène, quelle que soit la voie d'injection. La phase de plus grande sensibilité pour la production de cette malformation se place dans les 48 premières heures d'incubation, avec un maximum à 36 heures.

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The Association of Mucopolysaccharides with Morphogenesis of the Palate and Other Structures in Mouse Embryos

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WITH FOUR PLATES

ACID mucopolysaccharides have been postulated to occur in the palatine shelves of mouse embryos on the basis of metachromatic staining with toluidine blue (Walker & Fraser, 1956), and this idea has since been supported by results from radioautography with S^{35} (Larsson, Boström, & Carlsöö, 1958). It was not known which structural component of the tissue contained this substance, but, since aldehyde fuchsin-positive material existed in the same area, the possibility was raised that the component was a network of elastic fibres (Walker & Fraser, 1956). The first purpose of the present paper is to describe further histochemical and radioautographic investigations of this problem, supplemented by electron microscopic observations of palatine shelf tissue.

Numerous sites of S^{35} incorporation have been identified in embryos of various ages, including embryos too young to contain cartilage (Dziewaitkowski, 1958). Very little was concluded from such studies concerning the role of sulphated acid mucopolysaccharides other than noting their characteristic presence as a component of cartilage and bone. In view of the apparent association between sulphated acid mucopolysaccharides and palate development (Walker, 1960), it seemed desirable to gain a broader perspective on the possible significance of this substance in morphogenesis, so that the experiments described in the present paper were extended secondarily to include earlier stages of embryonic development.

MATERIALS AND METHODS

Timing of embryos was by vaginal plug initially and was confirmed later by reference to morphological criteria. The details of mating and timing procedures have been described previously (Walker & Fraser, 1956). The histochemical methods used were aldehyde fuchsin (Pearse, 1953), toluidine blue (Pearse, 1953), alcian blue (Mowry, 1956), and orcinol-new fuchsin (Fullmer & Lillie, 1956).

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The fixative used was Bouin's fluid in all cases. Embryos were collected at regular intervals from 7 to 17 days post-conception for staining with aldehyde fuchsin, alcian blue, and toluidine blue, but orcinol-new fuchsin was applied only to heads from 15-day-old embryos. Amniotic fluid was withdrawn from embryos at days 14/8 (14 days and 8 hours post conception), 15/8, 17/8, and 19/8. The fluid was smeared on slides, fixed in methanol or Bouin's fluid, and stained with aldehyde fuchsin.

Palate tissue used for electron microscopy was dissected from the living embryo, placed in cold buffered osmic acid sucrose fixative (Caulfield, 1957) for 45 minutes, and then dehydrated and embedded in methacrylate.

For radioautography of the palatine shelves, 1·5 mc. of S^{35} (as $Na_2S^{35}O_4$) was injected into a pregnant mouse in doses of 0·5 mc. at days 12/10, 13/8, and 14/8, while another mouse received 1·5 mc. S^{35} (in four smaller doses) at days 14/6, 14 10, 14/14, and 14/18. Both were killed at day 15/9. Also, single doses of 180 μc. S^{35} were given at days 13/14 and 14/10 to two pregnant mice, and these mice were killed at days 14/14 and 15/10 respectively.

For radioautography of young embryos 1·0 to 1·5 mc. of S^{35} were administered to four pregnant mice in one to three doses at various intervals starting at day 9 7. These animals were killed at days 9/16, 10 6, or 10/10. Embryos were fixed in Bouin's fluid and the radioautographs were made by the fluid emulsion dipping method which has been described elsewhere (Walker, 1959). Most of the radioautographs were exposed for 3 months; after development, some were stained lightly with eosin.

Experiments *in vivo* with palatine shelf movement were based on the selection of embryos at the proper stage of development, which was determined by the vaginal plug method and then confirmed by observing shelf behaviour in a few of the embryos from each litter used. The procedure for *in vivo* experiments has been described elsewhere (Walker, 1954; Walker & Fraser, 1956). The treatments applied were (*a*) hyaluronidase (150 turbidity reducing units in 2 c.c. H_2O for 10 minutes to 2 hours at 37° C., with water controls), (*b*) 1/100 N and 1 N solutions of HCl or KOH, and (*c*) various mixtures of acetic acid and alcohol. The experiments were carried out on relatively small numbers of embryos, because the results were not encouraging enough with any of these agents to warrant a large series.

RESULTS

Mesenchyme of the palatine shelves, when viewed with the electron microscope, appears to consist of cells with long processes embedded in an extensive matrix of intercellular ground substance (Plate 1, fig. A). Mitochondria and a small amount of granular endoplasmic reticulum are present within the cytoplasm. At least some of the cytoplasmic processes of each cell come in contact with those of adjoining cells (Plate 1, fig. B). No elastic fibres were found in the

mesenchyme of the palatine shelves. Also, the oral epithelium did not appear to contain any specialized structures (Plate 1, fig. C).

The location and intensity of aldehyde fuchsin staining reactions and of S^{35} incorporation in sections of 15-day-old embryo heads are illustrated in figs. D and E of Plate 2 and in figs. I and J of Plate 3; they are also listed in Table 1.

TABLE 1

Location and intensity of histochemical reactions in 15-day-old embryo heads

Histochemical agent	*Connective Tissue*				*Other Structures*				
	In palate	*In eyelids*	*Under oral epithelium*	*Under neural epithelium*	*Cartilage*	*Bone*	*Mast cells*	*Oral epithelium*	*Neural epithelium*
S^{35}	++	+	+++	+++	+++	+++	+++	—	—
Aldehyde fuchsin	++	++	++	++	+++	+++	+++	—	—
Toluidine blue metachromasia	++	+	+	+	+++	+	++	—	—
Alcian blue	++	++	++	++	+++	++	++	—	±
Orcinol-new fuchsin	—	—	—	—	—	—	—	—	—

TABLE 2

Aldehyde-fuchsin staining in connective tissue of mouse embryos

Location of connective tissue	*Age of embryo (days)*			
	8	9	10	11
Bordering neural epithelium	++	++	++	+++
Around lens			++	+++
In nasal and maxillary processes		±	+	+++
In cardiac jelly	++	++		
In bulbus			++	++++
In endocardial cushions			+++	++++
In interatrial septum				+++
In large blood-vessels	+	++	++	+++
In dorsal mesentery	++	++	++	++++
Around U.G. system			+	+++
In roof of pharynx	++	++	++	++++
In notochord sheath	++	++	++	++++
In limb-buds		—	—	++
Around somites	++	++	++	+++

A similar survey was made on sections of both the head and body of younger embryos and the results are illustrated in the remaining figures of Plates 3 and 4 and are summarized in Table 2. Seven-day-old embryos are not listed in Table 2 because these early post-implantation embryos gave essentially negative results. Alcian blue and toluidine blue were also used to stain young embryos and the results were approximately the same as with aldehyde fuchsin, although the weaker reactions were not as easy to identify.

All samples of amniotic fluid collected gave a positive reaction with aldehyde fuchsin. The material was precipitated as strands, loosely associated, to form a network in which cells were embedded. Many of these cells also gave a positive staining reaction. The presence of radioactive or aldehyde fuchsin-positive material on epithelial surfaces (Plates 2, fig. E; 3, fig. G) may be due to the precipitation of this material from the amniotic fluid.

Observations on shelf movement in 'living' embryos were made under a variety of experimental conditions. Incubation at 37° C. for 2 hours in distilled water did not inhibit palatine shelf movement. Sections made after an hour of such treatment showed swelling of the nuclei and a marked reduction in the density of their staining. Average diameters ($\sqrt{\text{longest axis} \times \text{maximum diameter at } 90°}$) were calculated for 40 nuclei in the palate mesenchyme and they averaged 6·5 μ, whereas the corresponding figure in a control embryo was 5·4 μ. Hyaluronidase may have had a slight inhibitory influence under these same conditions of incubation, but in most cases the shelves were still able to move after exposure to this enzyme. HCl at a concentration of 1/100 N did not inhibit shelf movement, whereas 1 N HCl not only stopped shelf movement, but tended to harden the whole embryo; the action of this agent on shelf movement is therefore not necessarily different from its action on tissues in general. The same was true for the other reagents used. Thus, no agent specifically inhibitory to shelf movement was demonstrated by these experiments.

DISCUSSION

At the time of normal palate closure, the palatine shelves are able to move from a vertical to a horizontal plane due to some force residing within the shelves (Walker & Fraser, 1956). In a search for the physical basis of this force with the electron microscope, no unique structural specialization was found in the thin epithelium or loose mesenchyme of the palatine shelves (Plate 1, figs. A–C). Accordingly, the force would seem most likely to reside in the ground substance, or in the network of mesenchyme cells, or in the vascular system. The latter has already been ruled out by demonstrating that shelf force is independent of blood-pressure and that it even is resistant to immersion in 70 per cent. alcohol for 15 minutes (Walker & Fraser, 1956). The *in vivo* experiments reported here were not described in detail because they did not lead to the identification of reagents affecting palatine shelves specifically. They were included just to emphasize the resistance to noxious agents exhibited by the force which caused shelf movement. Since the palatine shelves can still move after exposure to weak acid solutions or after immersion in distilled water at 37° C. for 2 hours, it seems probable that the final release of this force is not immediately dependent on cytoplasmic activities, and that it should be attributed to some material produced by the cells prior to these treatments. The extensive network of ground substance in the mesenchyme is the most likely site for this

material, considering the morphology of the shelves as seen with the electron microscope.

The orcinol-new fuchsin stain introduced by Fullmer & Lillie (1956) is specific for elastic fibres. When used on sections from 15-day-old embryo heads, it did not stain any of the tissues present (Table 1). Since the electron microscope also did not reveal any elastic fibres, the hypothesis that these latter structures are responsible for the shelf force must be discarded. This hypothesis was originally based on the presence of aldehyde-fuchsin positive material in the palate mesenchyme, so that the problem which now remains is to determine what is being stained by this reagent. A comparison of figs. D and E in Plate 2 shows the exceptionally close parallel between aldehyde fuchsin staining and S^{35} incorporation. There is an extensive literature (e.g. Boström, 1958; Dziewiatkowski, 1958; Friberg, 1958) associating S^{35} incorporation with sulphated acid mucopolysaccharide synthesis when the S^{35} is administered as sodium sulphate. Furthermore, there is a close parallel between the reactions produced by these reagents and the reactions of two stains widely used for acid mucopolysaccharides, namely, toluidine blue (metachromasia) and alcian blue (Table 1). Thus it seems reasonable to conclude that the ground substance of the palatine shelves contains a considerable amount of sulphated acid mucopolysaccharide. There is probably more than one type of mucopolysaccharide present in the ground substance, as it is believed that these various histochemical stains cannot all react to the same mucopolysaccharide (Spicer, 1960; Fullmer, 1960).

The considerably greater intensity of palatine tissue radioactivity in radioautographs derived from embryos receiving S^{35} from day 14/8 to 14/18 (Plate 2, fig. B) than from embryos receiving S^{35} from day 12/10 to 14/8 can be considered as evidence associating sulphated mucopolysaccharide synthesis with the build up of palatine shelf force, since the latter phenomenon is known to take place mainly from day 14/8 to 14/20 (Walker, 1954).

A few studies on young embryos have been reported using S^{35} (Dziewiatkowski, 1958) and histochemical stains for mucopolysaccharides (Milaire, 1959). The survey study with S^{35} and aldehyde fuchsin reported here is not meant to be comprehensive but has had as its main objective a further test, with these two techniques, of the apparent parallel distribution of positive reactions in embryonic tissues. The aldehyde fuchsin was applied to a greater range of developmental stages than was S^{35}, due to the simplicity of the former technique, but in all cases where the two methods were compared, the distribution and intensity of reactions were essentially the same.

A discussion of the significance of this material in morphogenesis is necessarily speculative at this time, but certain features of the embryological events with which the mucopolysaccharides are associated seem worth commenting on in view of the physical properties of these compounds. The distribution of sulphated acid mucopolysaccharide in tissues is mainly in structures having the consistency of a firm gel (Ham, 1957). More specifically, chondroitinsulphuric

acid can be combined with certain proteins in the test tube to produce a 'coherent stringy elastic mass' with an 'appearance not unlike elastic fibres' or 'resembling the ground substance of bone', depending on the protein used (Meyer, Palmer, & Smyth, 1937). The only modification necessary to enable such a substance to bring about movement of the palatine shelves is that it should not be in its conformation of maximum stability prior to the actual movement. This instability might be due to asymmetrical deposition and subsequent growth stresses, to growth pressures from external sources, or to chemical alteration of the material itself. Whether such mechanisms are possible, or whether any of them exist in the embryo, is not known at present. Nevertheless, such an hypothesis is attractive because it could explain not only palatine shelf movement but also a number of other morphogenetic events.

Considering the intense reaction of the notochord sheath and the moderate reactivity of the surrounding mesenchyme (Plate 3, fig. K), the concept of a resilient system under pressure has obvious application to the phenomenon described in the following quotation (Snell, 1941): 'At about 8½ days, and at about the 11 or 12 somite stage, the mid-trunk region turns also. The process is sudden. Transverse sections of the trunk region at about this period show it to be either turned or not turned. It is quite possible that after the growth of the head and tail folds reduces sufficiently the attachment of the trunk region to the yolk sac, this region snaps over like a spring whose tension has come to exceed the forces holding it.'

Closure of the neural tube is another event with an obscure mechanism (Weiss, 1955). Arguments against differential cell-growth or cell proliferation have been reinforced by recent demonstrations of cyclic nuclear migration between the luminal and peripheral areas of the tube (Sauer & Walker, 1959; Sidman, Miale, & Feder, 1959). The force producing a folding of the neural tube was believed to reside within the tube because excised and isolated plates can transform into tubes (Weiss, 1955). Inspection of Plate 4, fig. O will show that the mucopolysaccharide layer enveloping the neural tube (which is also present when the tube is in the process of closing) lies at the epithelial-connective tissue junction and is most unlikely to have been separated off in the experiments referred to by Weiss. The neural tube itself may be contributing to this layer, especially since the epithelial cells of the enamel organ that form the stellate reticulum are apparently able to produce this type of mucopolysaccharide (Plate 3, fig. J).

Another embryological process in which mucopolysaccharides may participate is lip formation. According to Reed (1933), the maxillary process must press tightly against the nasal processes if the lip is to form properly. There is a considerable amount of aldehyde fuchsin-positive (Plate 3, figs. F, G) and S^{35}-containing (Plate 4, fig. O) material in these processes by the time they are ready to fuse.

The progressive development of cardiac jelly into heart-valves proposed by

Patten, Kramer, & Barry (1948) is paralleled by the appearance of mucopolysaccharides in cardiac jelly (Plate 4, fig. Q) and later in septa of the heart (Plate 4, fig. N) and bulbus (Plate 4, fig. M). Also, the property of resiliency suggested here for this type of mucopolysaccharide is consistent with the function attributed to cardiac jelly by Barry (1948).

The presence of mucopolysaccharide in the dorsal mesentery (Plate 4, fig. L), and especially where it extends into the umbilical cord (Plate 4, fig. P), can be tentatively ascribed to a role of support. Finally, concentrations of reactive material in areas where epithelia have abrupt or elaborate contours (Plate 3, figs. H, I) may be associated with moulding or support, the latter function having been proposed already by Patten *et al.* (1948) for some gelatinous material like cardiac jelly.

It is perhaps worth emphasizing that the reason for suspecting an important relationship between sulphated mucopolysaccharides and the embryological events referred to above is not simply because this material is present but because the time at which it appears and the locations where it reaches the highest concentration are associated with events which are easiest to explain by assuming the involvement of a firm, elastic gel, such as the gels in which sulphated mucopolysaccharides can be found. Certainly, the exact basis for the movement of palatine shelves remains obscure, but further study of the sulphated mucopolysaccharides appears to be the most promising approach at present.

The potential application of the foregoing to experimental teratology is obvious when one considers the frequency with which the embryological processes just discussed are affected by teratogens like cortisone (Fraser & Fainstat, 1951; Walker & Crain, 1959), hypervitaminosis A (Kalter & Warkany, 1959), and vitamin A deficiency (Wilson & Warkany, 1949), all of which are known to interfere with the metabolism of mucopolysaccharides (Boström, 1958; Dziewaitkowski, 1958; Fell, 1956). Similarly, Runner (1959) has related the action of several teratogens (which affected, primarily, the morphogenesis of embryonic neural tube and the differentiation of precartilaginous mesenchyme) to disturbances in carbohydrate metabolism; the latter can then be related to mucopolysaccharide synthesis (Whistler & Olson, 1957).

SUMMARY

1. Palatine shelf tissue was taken from mouse embryos at the stage of palate closure and studied with the electron microscope. The mesenchyme consisted of cells with long cytoplasmic processes embedded in a considerable quantity of ground substance, but no connective-tissue fibres were present. The oral epithelium covering this mesenchyme had no obvious structural specializations.

2. Sections of 15-day-old embryo heads were studied with various histochemical techniques and by radioautography after administration of $Na_2S^{35}O_4$. The distribution of aldehyde fuchsin-positive material corresponded to sites of

S^{35} incorporation. No positive reactions were obtained with the orcinol-new fuchsin elastic fibre stain.

3. Earlier stages of embryogenesis were studied with mucopolysaccharide stains and with S^{35} radioautography. Again, the distribution of aldehyde fuchsin-positive material corresponded closely to the areas of S^{35} incorporation.

RÉSUMÉ

L'Association de mucopolysaccharides avec la morphogenèse du palais et d'autres structures chez l'embryon de Souris

1. Du tissu des lames palatines est prélevé sur des embryons de Souris au stade de la fermeture du palais, et il est étudié au microscope électronique. Le mésenchyme consiste en cellules à longs prolongements cytoplasmiques enrobés dans une quantité considérable de substance fondamentale, mais on ne trouve pas de fibres de tissu conjonctif. L'épithélium oral qui couvre ce mésenchyme ne montre pas de structure spécialisée.

2. Des sections de têtes d'embryon de 15 jours ont été étudiées au moyen de techniques histochimiques variées et par autoradiographie après administration de $Na_2S^{35}O_4$. La distribution du matériel positif à la fuchsine-aldéhyde correspond aux niveaux d'incorporation de S^{35}. Aucune réaction positive n'a été obtenue avec coloration des fibres élastiques à la fuchsine néo-orcinol.

3. Des stades plus jeunes de l'embryologie ont été étudiés à l'aide des colorants de mucopolysaccharides et par autoradiographie du S^{35}. Ici encore, la distribution du matériel positif à la fuchsine-aldéhyde correspond exactement aux aires d'incorporation de S^{35}.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES

Plate 1

Fig. A. Electron micrograph of mesenchyme from the palatine shelves. This tissue consists of cells with long processes and an extensive intercellular area. The site of S^{35} incorporation and aldehyde fuchsin reaction is believed to be the intercellular area. × 3800.

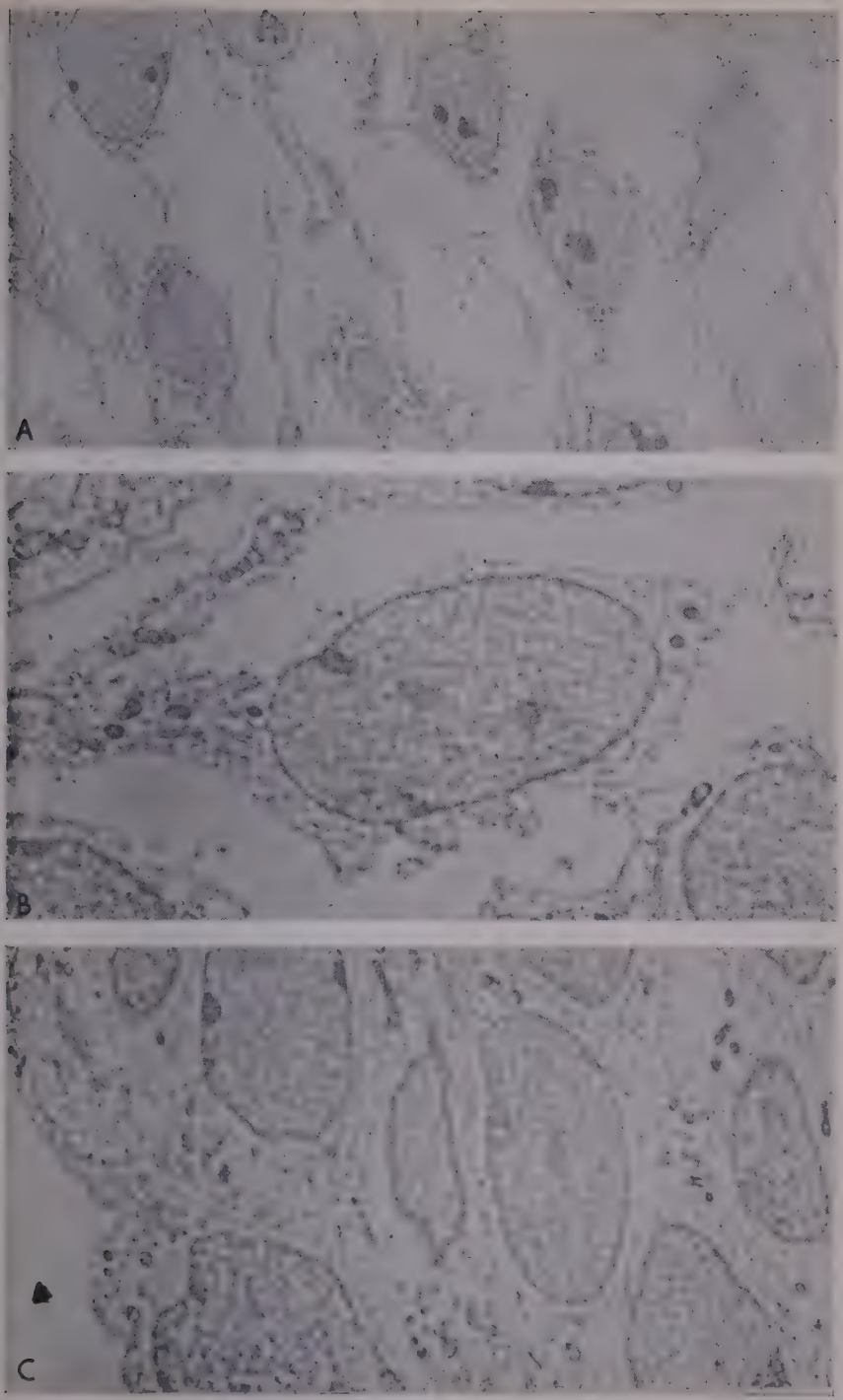
Fig. B. Electron micrograph of palate mesenchyme at higher magnification. The mesenchymal cell contains mitochondria and a small amount of granular endoplasmic reticulum. There are no elastic or collagenous fibres in the intercellular substance. × 8600.

Fig. C. Electron micrograph of oral epithelium from the palatine shelves. No special structural elements have been identified in this tissue which could be associated with the mechanism of palate closure. × 5400.

Plate 2

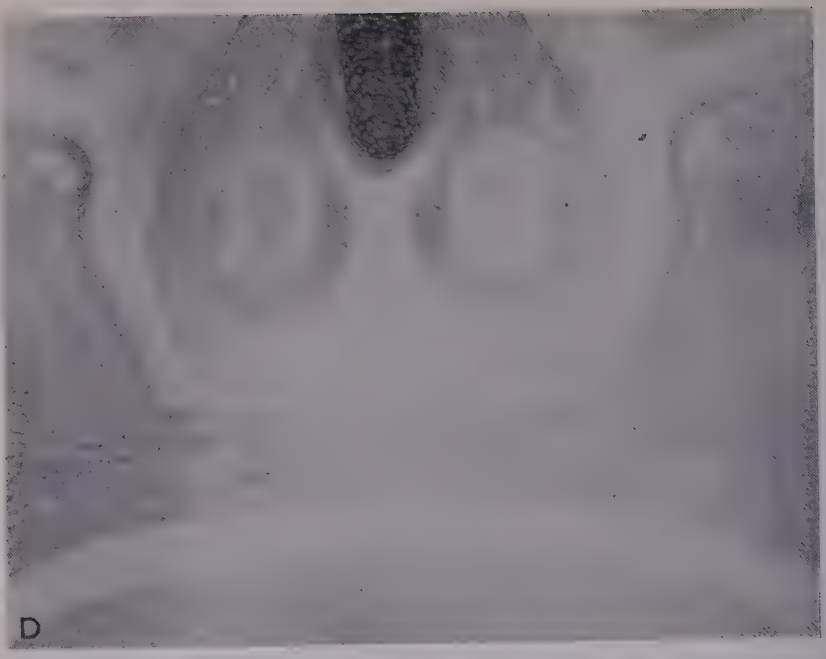
Fig. D. A section through the head in the region of palate and nasal septum, taken from an embryo fixed shortly after closure of the palate. Aldehyde fuchsin-positive material is present in cartilage, at the epithelial-connective tissue boundaries, and in the form of a network throughout the palatine shelves. × 97.

Fig. E. Unstained radioautograph of a section through the head of an embryo taken from the



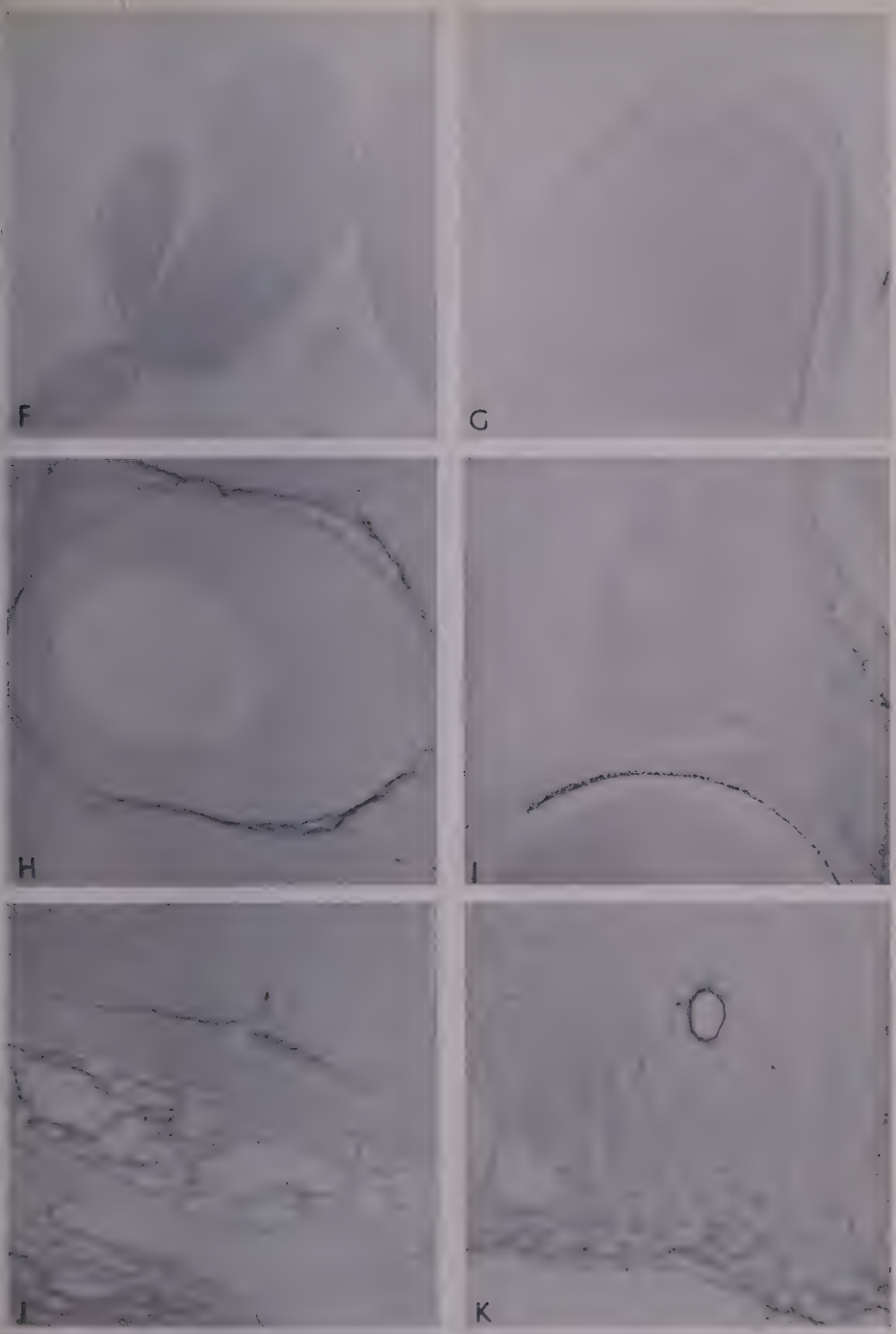
B. E. WALKER

Plate 1



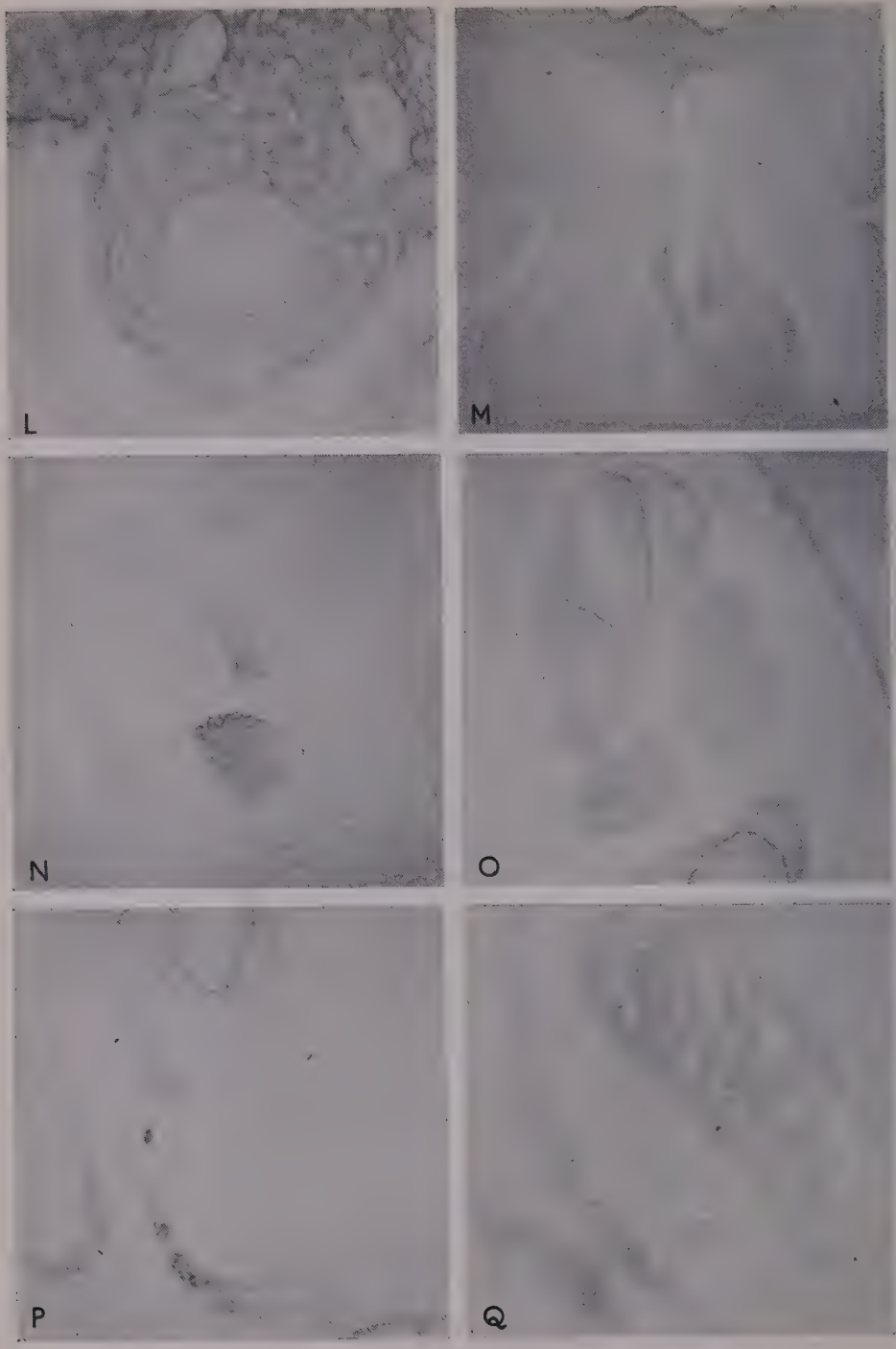
B. E. WALKER

Plate 2



B. E. WALKER

Plate 3



B. E. WALKER

Plate 4

same litter as the one represented in fig. D. Notice the exceptionally close parallel between the distribution of aldehyde fuchsin and S^{35}, both qualitatively and quantitatively. The dark reactions at the sides of the photo are over areas of membrane bone formation (which are not included in fig. D although they give a dark reaction with aldehyde fuchsin also). ×91.

Plate 3

Sections from embryos of various ages stained with aldehyde fuchsin only.

Fig. F. Maxillary and nasal processes at the time of lip formation. The connective tissue gives a positive reaction. (The aldehyde fuchsin reactions are difficult to demonstrate photographically but the original colour distinctions are clearly defined in the slides.) ×51.

Fig. G. The maxillary process at higher magnification. The distribution of stainable material as a network in the mesenchyme resembles that of the palatine shelves. The layer of stained material on the epithelial surface is probably derived from precipitation of the aldehyde fuchsin-positive material in amniotic fluid. ×320.

Fig. H. A strong reaction outlines the lens and optic cup of the developing eye. ×260.

Fig. I. Sharp contours of epithelium associated with eyelid and hair follicle are outlined by concentrations of aldehyde fuchsin-positive material in the mesenchyme. (Small areas of membrane bone formation to the extreme right give a very dark reaction.) ×64.

Fig. J. The border of the enamel organ and material in the stellate reticulum stain strongly. The stellate reticulum is believed to be produced by cells of epithelial origin. ×310.

Fig. K. The sheath of the notochord (top, centre) stains intensely with aldehyde fuchsin and is surrounded by mesenchyme containing a considerable amount of stainable material (lumen of pharynx is at the bottom of the photo). ×280.

Plate 4

Fig. L. Gut (centre) and blood-vessels surrounded by a network of aldehyde fuchsin-positive material in the dorsal mesentery. ×290.

Fig. M. Section through bulbus and aortic arches. The bulbar cushions have a large quantity of aldehyde fuchsin-positive material. ×67.

Fig. N. The septum primum (centre) and endocardial cushion (lower centre) contain dense masses of material stained with aldehyde fuchsin. ×67.

Fig. O. Radioautograph of an unstained section through the brachial arches and neural tube of an embryo which received S^{35}. The epithelial-connective tissue junctions around the neural tube and in the arches are radioactive, as is the connective tissue of the arches. The embryonic membranes are radioactive too. ×65.

Fig. P. Radioautograph of a section through the abdominal region of an embryo which received S^{35}. The dorsal mesentery, extending into the umbilical cord with the gut, contains a large amount of radioactive material. ×68.

Fig. Q. Radioautograph of a portion of the heart-tube at high magnification and stained lightly with eosin. The cardiac jelly between the outer wall and lumen (filled with erythrocytes) is overlaid by silver grains which are distributed in a manner similar to the material precipitated from cardiac jelly that is stained by aldehyde fuchsin. ×340.

(Manuscript received 1 : vi : 60)

The Blood-Vessels of the Developing Spinal Cord of *Xenopus laevis*

by R. T. SIMS[1]

From the Department of Anatomy, University of Cambridge

WITH ONE PLATE

INTRODUCTION

STERZI (1904) studied the blood-vessels of the developing spinal cord in representatives of various vertebrate groups. He correlated the early development of the vascular plexus on the lateral aspect of the neural tube with the mitotic activity within its lateral walls. He also correlated the greater vascularity of the grey matter, compared with that of the white matter, with the greater functional activity of the former. From the observation that there are 15 separate vessels that are constant in position and time of appearance during the development of the spinal cord of the chick, Feeney & Watterson (1946) reached the tentative conclusion that the pattern of the blood-vessels is determined by localized structural or physiological changes, or both. Observations on mammals by Craigie (1925), Petren (1938), and Gyllensten (1959) indicated a marked increase in vascularity of the cerebral cortex while differentiation was proceeding. Quantitative observations on the blood-vessels of the spinal cord during development are lacking.

Craigie (1945) and Feeney & Watterson (1946) indicated the need for an experimental approach to the development of blood-vessels in the central nervous system. The present investigation was undertaken to establish the descriptive and quantitative features of the development of the blood-vessels of the spinal cord in an animal readily available in the laboratory as a foundation on which future experiments might be based.

MATERIAL AND METHODS

Tadpoles of *Xenopus laevis* were obtained, reared, and staged by the methods recommended by Nieuwkoop & Faber (1956). Specimens were taken at each stage and anaesthetized with M.S. 222. The vascular system was then injected with Monastral fast blue (B.N.V.S., Imperial Chemical Industries Ltd.) paste diluted with distilled water to a 1 in 5 solution. The injection was made with a fine glass micropipette into the third aortic arch of one side in stages 45–59, and into the

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atrium in stages 60–66. The aortic arches of the later stages were too thick to be pierced by a glass micropipette. In order to ensure complete injection the dye was introduced slowly into the blood-stream and allowed to mix thoroughly with the circulating blood while the heart was still pumping. As soon as the heart ceased to function the injection was stopped. It was not found possible to perform reliable complete injections at stages earlier than stage 45.

After injection the whole tadpole was fixed for 24 hours in an aqueous solution of 5 per cent. formalin and 0·5 per cent. glacial acetic acid. The spinal cord, together with the structures immediately surrounding it, was dissected out and dehydrated in ascending grades of alcohol, cleared in benzene, and embedded in paraffin wax. Some were bulk-stained with neutral red and others with alcoholic eosin. Serial sections were cut either transversely at a thickness of 100 μ, or sagitally and coronally at a thickness of 50 μ. For stages 45–53 a series in each of the three planes was cut for each stage and additional series were cut as follows: two transversely at stage 46; two transversely, one coronally, and one sagittally at stage 47; and five transversely at stage 49. For stages 54–66 only one transverse series at each stage was cut. The sections were dewaxed in xylol and mounted in balsam.

Sections prepared in this way allowed the vascular pattern to be examined in three dimensions with a stereoscopic dissection microscope. It was found that the particles of Monastral fast blue started to form long needle-shaped crystals about 3 months after mounting.

Monastral fast blue was injected into the atrium of one tadpole at stage 54 until the heart ceased pumping, and then a 1 in 5 solution of Monastral fast green (G.V.S., Imperial Chemical Industries Ltd.) paste in distilled water was injected into the 3rd aortic arch until it filled the systemic arteries. The animal was fixed and the central nervous system dissected out as before and bulk-stained with alcoholic borax carmine. After embedding in paraffin wax serial transverse sections were cut at a thickness of 25 μ. In this preparation arteries were filled with a green mass, veins with a blue mass, and the cells were stained red.

The direction of blood-flow in large vessels was clearly visible with the aid of a dissection microscope in the anaesthetized tadpoles at the earlier stages studied, and observations made in this way are included in the description of the findings.

QUANTITATIVE METHODS

For the purpose of this work the shape of a transverse section of the spinal cord was regarded as an ellipse and the central canal was ignored. The volume of cord segment in which the counts were made was calculated using the formula vol. $= \pi \frac{v}{2} \frac{t}{2} l$, where v = vertical diameter, t = transverse diameter, and l = length of cord.

Examination of the sections indicated that the vascular network increased in complexity by the addition of new capillary segments to those already present

by dichotomous divisions and junctions. If a segment is defined as the unbranched length of vessel between two points of division or junction, the number of segments is an index of the stage of development of the network. It must be emphasized that it is not an estimate of vascularity, i.e. of the length of vessel per unit volume. Attempts to count the number of vascular segments showed that it was easier to count the number of divisions and junctions and then to calculate the number of vascular segments using the formula $n = E+2A+B$, where n is the number of vascular segments, A the number of divisions, B the number of junctions, and E the number of entering arterial vessels.

The centrifugal nature of the blood-flow in the spinal cord of Anura allowed the counts to be made in the direction of flow from entering arterial vessels towards the periphery. A division was taken as a point where one vessel becomes two, and a junction as a point where two vessels converge to form one. For vessels running parallel to the long axis of the cord, those passing cranially from a transverse vessel were regarded as leaving a division and those passing caudally from a transverse vessel were regarded as leaving a junction. The formula was derived by arguing that the number of vascular segments in a network is equal to the number of entering arterial vessels, plus twice the number of divisions (each of which gives rise to two vascular segments), plus the number of junctions (each of which forms one vascular segment).

A count of these values was made at every stage from 49 to 66, inclusive, in three consecutive sections between the origins of the 2nd and 3rd segmental nerves, which supply the forelimbs, and at stages 56, 61, and 66 in three consecutive sections between the origins of the 5th and 6th, and 8th and 9th segmental nerves, which supply the trunk and hind limbs respectively. At the same time the mean of the vertical diameters and the mean of the transverse diameters of the spinal cord, measured with a 1-mm.^{2} graticule in the focal plane of the eye-piece, were found. The number of vascular segments in a volume of 1 mm.^{3} of spinal cord was then calculated for each stage of development.

Drawings of the same sections were made at a magnification of $\times 200$ with the aid of an Edinger projection apparatus at every stage from 52 to 66. After checking the accuracy of the drawings the combined lengths of all the vessels and parts of vessels were measured on the drawings with a map-measuring instrument. The length of vessel in mm. in a volume of 1 mm.^{3} of spinal cord was then calculated for each stage of development, and thus an estimate of vascularity was obtained.

OBSERVATIONS

Superficial vessels

At stage 45 branches of dorsal intersegmental arteries pass to the groove formed on each side between notochord and neural tube. On reaching the

groove they divide into cranial and caudal branches which join with the relevant adjacent vessel to form a longitudinal anastomotic vessel. At intervals branches from the longitudinal vessel pass dorsally over the lateral surface of the neural tube until they reach its dorso-lateral region, where in turn they divide into cranial and caudal branches, which join the relevant neighbouring vessel to form a series of arcades. From these arcades vessels pass to the veins of the body-wall. The dorsal direction of the blood-flow in these vessels was seen in the anaesthetized tadpole at this stage.

By the next stage a single median vessel has appeared on the dorsal surface of the cord, into which the vessels of the lateral surface drain. This vessel will be referred to as the 'dorsal spinal vein'. In the anaesthetized tadpole the blood in it was seen to flow cranialwards into the choroid plexus of the 4th ventricle. No direct arterial supply to this plexus was found in this material.

Between stages 46 and 47 a single median vessel is established on the ventral surface of the cord, and this will be referred to as the 'ventral spinal artery' (Plate, figs. A, B). No transitional stages were found to indicate its method of formation. Large vessels from the dorsal intersegmental arteries to the ventral spinal artery accompany the first segmental nerves of both sides and the 4th segmental nerve of one side; the vessels accompanying the other spinal nerves are small. The dorsal spinal vein at stage 47 has established connexions, which accompany the 5th and 10th spinal nerves, to the body-wall veins. During this stage a vessel, which joins the plexus on the ectomeninx covering the saccus endolymphaticus, develops closely applied to the meningeal surface of the ectomeninx over the first four segments of the spinal cord. In the anaesthetized tadpole the blood in this vessel was seen to flow cranially into the plexus over the saccus. This vessel will be referred to as the 'dorsal dural vein'.

By stage 49 the large vessels on the surface of the spinal cord have acquired the pattern found in the adult. The ventral spinal artery is situated in a shallow ventral median fissure and receives large arteries which accompany the first spinal nerve on both sides, and yet another large artery which accompanies the 5th or 6th spinal nerve on one side.

From the ventral spinal artery the blood is conducted over the lateral surface of the cord through a wide-meshed plexus to the dorsal spinal vein.

The dorsal dural vein (Plate, fig. F) extends over the entire trunk region of the cord and receives blood from the dorsal spinal vein, and from the plexus on the surface of the cord by vessels arising from the region in which the rootlets of the dorsal roots enter the cord. At this stage the saccus endolymphaticus is not situated over the spinal cord.

Between stages 54 and 58 the saccus on each side extends caudally as far as the 4th spinal nerve. The dorsal dural vein is not separated from the dura as the sacci extend alongside it (Plate, fig. G). The vessels from the spinal cord to the dorsal dural vein pass ventrally to the sacci. The specimen at stage 54 injected with two different colours showed that at this stage the plexus on the lateral

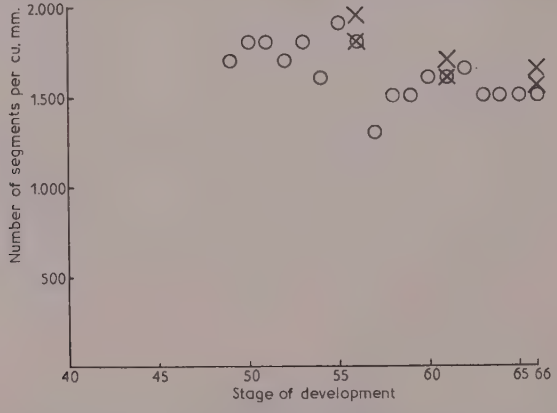
surface receives blood only from vessels within the cord and none from the ventral spinal artery, there being no other arterial supply to the spinal cord.

Vessels inside the spinal cord

The first vessels injected in the substance of the spinal cord were found in one of five specimens at stage 46, in five of seven specimens at stage 47, and in all specimens at later stages. They arise from the ventral spinal artery and enter the cord just lateral to the midline (Plate, fig. E). Then they pass dorsally to the plane between the ependymal and mantle layers at the level of the ventral limit of the spinal canal. In this situation they run cranially or caudally for a short distance before passing round the canal in the same plane to leave the dorsal surface in the midline. The first vessels are not paired, and the vascular pattern within the cord is asymmetrical.

At stage 48 a series of such vessels is present in the trunk region of the cord, and parts of a longitudinal anastomotic vessel have developed at the level of the ventral extremity of the spinal canal (Plate, fig. D). The longitudinal anastomotic vessel extends along the whole trunk region of the cord at stage 49 (Plate, fig. C). The vessels approaching the longitudinal vessel from the ventral surface are at different levels from those leaving it and running to the dorsal surface. The precise vascular pattern within the cord remains asymmetrical and inconstant from segment to segment and from specimen to specimen.

In the following stages new vessels appear which either run from existing vessels to those on the surface of the cord or run from one existing vessel to another. It is not possible to discern any individual vessels that are constant in position and time of appearance. Their only constant features are that branching and joining are dichotomous, and the vascular tree thus formed has

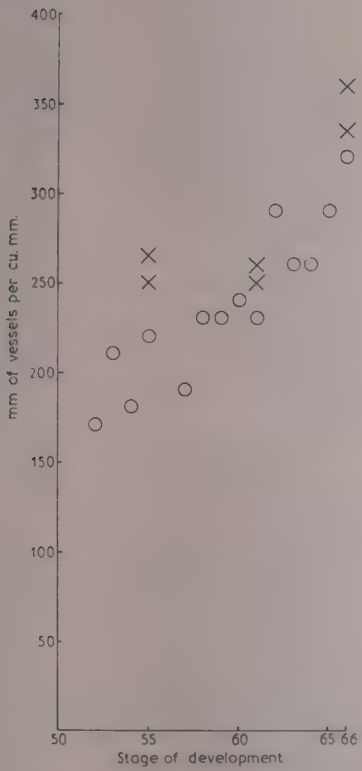


TEXT-FIG. 1. Graph showing the number of vascular segments per mm.^{3} in the developing spinal cord of *X. laevis* at each stage of development. ○, level of 2nd–3rd segmental nerves; ×, levels of 5th–6th and 8th–9th segmental nerves.

its trunk at the ventral median fissure, while its terminal branches leave the cord surface on all aspects to join the superficial plexus.

Quantitative observations

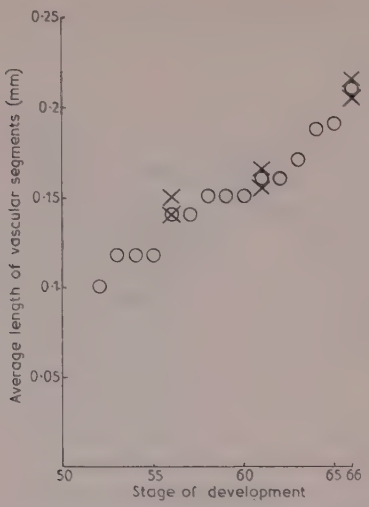
The number of vascular segments per mm.^{3} of spinal cord between the 2nd and 3rd segmental nerves at each stage of development is illustrated in the graph of Text-fig. 1. The points on the graph are scattered, but the number of vascular segments per mm.^{3} is slightly less for the later stages than for the earlier stages of development. The two additional estimates for each of stages 56, 61, and 66 are within 15 per cent. of the estimates for the level between the 2nd and 3rd segmental nerves.



TEXT-FIG. 2. Graph showing the combined length of vessels in mm. per mm.^{3} in the developing spinal cord of *X. laevis* at each stage of development. ○, level of 2nd–3rd segmental nerves; ×, levels of 5th–6th and 8th–9th segmental nerves.

The length of vessel per mm.^{3} of spinal cord at the level between the 2nd and 3rd segmental nerves for each stage of development from stage 52 to 66 is shown on the graph of Text-fig. 2. The values for stages 49–51 were not measured, as the volume of spinal cord involved was so small. Again the points are scattered, but there is a definite increase in vascularity as development proceeds. The two additional measurements at each of stages 56, 61, and 66 are within 15 per cent. of the measurements for the level between the 2nd and 3rd segmental nerves.

Inspection of these two graphs suggested that the average length of the vascular segments increases during development. The average length of the vascular segments was calculated by dividing the length of vessel per mm.^{3} by the number of vascular segments per mm.^{3} The results obtained are presented in the graph of Text-fig. 3. There is a gradual increase in the average length of the vascular segments, as development proceeds, to a value at stage 66 which is double that at stage 52. The amount of scatter of the points in the graph is less than in the previous graphs. The average length at any given stage is greater than the average length at any earlier stage and smaller than at any later stage. The values for the additional levels at each of stages 56, 61, and 66 are within 10 per cent. of the values for the level between the 2nd and 3rd segmental nerves.



TEXT-FIG. 3. Graph showing the average lengths of the vascular segments in mm. in the developing spinal cord of *X. laevis* at each stage of development. ○, level of 2nd–3rd segmental nerves; ×, levels of 5th–6th and 8th–9th segmental nerves.

DISCUSSION

The development in *Xenopus* of a pair of longitudinal vessels in the groove between the spinal cord and notochord, followed later by that of a single ventral spinal artery, resembles that described for *Rana esculenta* by Sterzi (1904). He found, however, that the dorsal spinal vein of *R. esculenta* became separated from the cord as the meninges develop, whereas in *Xenopus* the dorsal spinal vein is never separated from the cord and the dorsal dural vein appears as a separate entity growing caudally from the plexus over the saccus endolymphaticus.

The site at which the first vessel penetrates the cord of *Xenopus* and its course between ependymal and mantle layers resembles that for all other vertebrates, the Urodela excepted, as Sterzi (1904) emphasized. Its exit from the dorsal rather than the lateral surface may be associated with the relatively early development of a plexus over the dorsal surface of the cord.

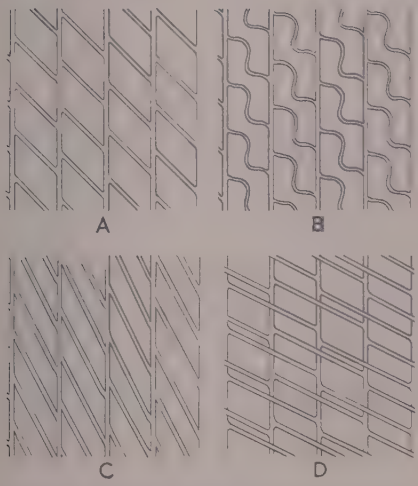
In *R. esculenta* Sterzi (1904) found two paired longitudinal anastomotic vessels, one at the level of the ventral limit and one at the level of the dorsal limit of the ependyma, while in *Xenopus* only one paired ventral longitudinal vessel was found. Feeney & Watterson (1946) found a similar anastomosis in the chick and suggested that there may be something peculiar about this part of the cord as it is so constantly associated with such a vessel. It is suggested

here that the plane between the ependymal and mantle layers is peculiar in being the region farthest away from the superficial vascular network.

The absence of individual vessels that are constant in position and time of appearance contrasts with the observations of Feeney & Watterson (1946) on the chick embryo. The differences in the external and internal environment and mode of life of the two creatures are so great that this contrast cannot be regarded as significant.

Discussion of quantitative observations

The observation that during development the degree of vascularity (in terms of length of vessel per unit volume of spinal cord) is increased while the number of vascular segments per mm.^{3} remains almost constant, leads to the finding that the average length of the vascular segments is increased. It is possible that this increase in average length occurred in one of three ways, or as the result of



TEXT-FIG. 4. Diagram showing three ways in which the vascularity of the spinal cord of *X. laevis* might be increased if the number of vascular segments per unit volume is constant and the average length of the vascular segments is increased. A, primary condition. B, vascularity increased by tortuosity. C, vascularity increased by decreasing the angle of divergence from parent stem with no increase in tortuosity. D, vascularity increased by passing other vessels with no increase in tortuosity.

the combination of several. If the capillary network is arranged so that any given vascular segment branches from one vessel and runs to join a neighbouring vessel without passing another vessel, then its length may be increased by increasing its tortuosity as in Text-fig. 4 A, B, or by decreasing the angle at which

it diverges from its parent stem (Text-fig. 4 A, C). Alternatively, if the capillary network is arranged so that any given vascular segment may arise from one vessel and pass other vessels to join a third vessel, then the increase in average length can occur without increasing the tortuosity of the vascular segment or decreasing the angle at which it diverges from its parent stem (Text-fig. 4 A, D).

On re-examination of the sections it was found impossible to assess whether increased tortuosity or decreased angle of divergence occurred. However, it was found that vascular segments which passed other vessels without joining them made their appearance at stage 55 and were present in all subsequent stages.

The increase in vascularity, in terms of length of vessel per unit volume of spinal cord, took place during the period of development in which the primary motor and sensory systems of the cord are replaced by their adult equivalent (Nieuwkoop & Faber, 1956; Hughes, 1957; Hughes & Tschumi, 1958; Hughes, 1959). A similar increase in vascularity has been shown to occur during the period in which the cerebral cortex differentiates in mammals by Craigie (1925), Petren (1938), and Gyllensten (1959).

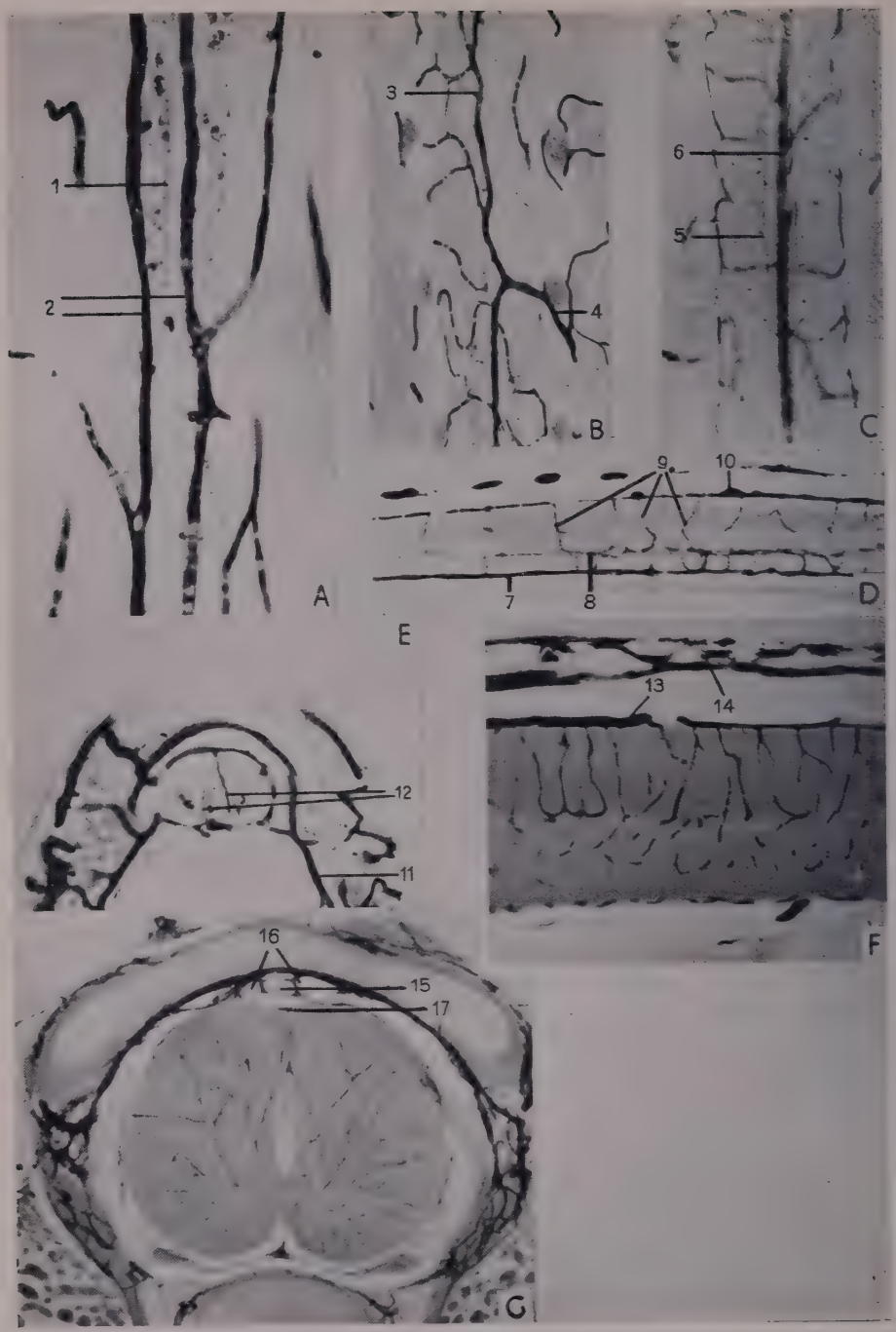
SUMMARY

The blood-vessels of the developing spinal cord of *X. laevis* have been studied from stage 45 to stage 66 by the injection technique. Within the substance of the cord the first vessels injected were found at stage 46, and individual vessels were not constant in position or time of appearance. The vascular network is formed by dichotomous branching and joining of vessels. Estimates of the number of vascular segments (i.e. between branches) per mm.^{3} of spinal cord and the total length of vessel per mm.^{3} showed that the latter increased during development while the former did not. It is concluded that the vascularity of the spinal cord increases as differentiation of the cord proceeds and is brought about by increasing the average length of the vascular segments.

RÉSUMÉ

Les vaisseaux sanguins de la moelle épinière au cours du développement de Xenopus laevis

Les vaisseaux sanguins de la moelle épinière de *Xenopus laevis* en voie de développement ont été étudiés entre les stades 45 et 66 par la technique des injections. A l'intérieur de la substance de la moelle, les premiers vaisseaux injectés ont été trouvés au stade 46; les vaisseaux individuels ne sont constants ni par leur position ni par leur ordre d'apparition. Le réseau vasculaire est formé par des branches dichotomes et des anastomoses. L'estimation du nombre de segments vasculaires (c'est-à-dire entre les branches) par mm.^{3} de moelle et de la longueur totale des vaisseaux par mm.^{3} montre que celle-ci augmente pendant le développement, mais non celui-là. On en conclut que la vascularisation de la moelle augmente en même temps que la différenciation progresse et qu'elle est causée par l'augmentation de la longueur moyenne des segments vasculaires.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EXPLANATION OF PLATE

Fig. A. Coronal section of tadpole of *X. laevis* at stage 46 showing spinal cord (1) and paired longitudinal anastomotic vessels (2) in groove between spinal cord and notochord. ×250.

Fig. B. Coronal section of tadpole of *X. laevis* at stage 47 showing the ventral spinal artery (3) and branch from the dorsal intersegmental artery (4). ×80.

Fig. C. Coronal section of tadpole of *X. laevis* at stage 49 showing paired longitudinal anastomotic vessels (5) within walls of spinal cord and ventral spinal artery (6) out of focus. ×80.

Fig. D. Sagittal section of tadpole of *X. laevis* at stage 48 showing ventral spinal artery (7), paired longitudinal anastomotic vessels (8) within walls of spinal cord (one is out of focus), and the variable course of the first vessels to enter the cord (9) passing round the central canal to the dorsal spinal vein (10). ×80.

Fig. E. Transverse section of tadpole of *X. laevis* at stage 46 showing dorsal intersegmental arteries (11) passing round notochord and giving branch to superficial vessels of spinal cord from which the first vessels to penetrate the spinal cord (12) arise. ×150.

Fig. F. Slightly oblique sagittal section of *X. laevis* at stage 54 showing the dorsal spinal vein (13) and dorsal dural vein (14). ×90.

Fig. G. Transverse section of *X. laevis* at stage 60 showing dorsal dural vein (15) flanked by sacci endolymphatici (16) with the dorsal spinal vein (17) below it. ×45.

(*Manuscript received 7 : vi : 60*)

The Differential Growth Response of Embryonic Chick Limb-bone Rudiments to Triiodothyronine *in vitro*

1. Stage of Development and Organ Size

by KIRSTIE LAWSON[1, 2]

From the Strangeways Research Laboratory, Cambridge

INTRODUCTION

THE proportionate development of the embryonic chick skeleton can be influenced experimentally by a variety of factors such as nutritional deficiencies (Byerly, Titus, Ellis, & Landauer, 1935; Landauer, 1936; Romanoff & Bauernfeind, 1942; Couch, Cravens, Elvehjem, & Halpin, 1948), teratogens (Ancel & Lallemand, 1942; Zwilling & de Bell, 1950; Landauer, 1952, 1953*a*, 1954) and excess hormones (Willier, 1924; Landauer & Bliss, 1946; Duraiswami, 1950). The leg bones are generally more severely affected than the wing bones, but a comparison of the action of several teratogens on the character of the malformations and on the relative growth of the leg bones indicated that the response of individual bones varies with the different agents (Landauer & Rhodes, 1952; Landauer, 1953 *a*, *b*, 1954).

Cartilaginous limb-bone rudiments also respond differentially when they are isolated from the embryo and exposed in culture to various compounds, such as insulin (Chen, 1954), vitamin A, and the thyroid hormones (Fell & Mellanby, 1955, 1956). Each agent has a characteristic histological effect, but the different rudiments are affected to a different extent. Maturation of the cartilage is accelerated in all the long-bone rudiments grown in the presence of additional thyroxine (T_4) or triiodothyronine (T_3), but the growth response varies; thus the growth in length of the tibia is severely retarded by the hormones while that of the radius is increased. Fell & Mellanby concluded that these differences in response were correlated with differences in the rate of normal diaphyseal differentiation in the different rudiments, and also with the stage of development at which the rudiments are exposed to the hormones.

This differential response of limb-bone rudiments to T_3 in culture has been analysed further, since it presumably reflects metabolic differences which may affect the development of the normal proportions of the skeleton. This paper

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reports experiments on the influence of developmental stage and organ size on the growth response to T_3. In subsequent papers the effect of variations in growth rate and hormone concentration on the differential response will be considered.

MATERIALS AND METHODS

Tissue culture

Cartilaginous limb-bone rudiments from 5- to 8-day-old chick embryos were cultivated in watch-glasses on a medium of fowl plasma and chick embryo extract (Fell & Robison, 1929; Fell & Mellanby, 1952). Embryo extract was prepared from 13- or 14-day-old embryos (Fell & Mellanby, 1952). One part of embryo pulp was extracted with one part of 1 per cent. glucose Tyrode; one part of the extract was then mixed with three parts of plasma.

0·5 mg. of the sodium salt of 3:5:3′ triiodo-L-thyronine (T_3) was weighed with sterile precautions and dissolved in 16 ml. of sterile 0·1 per cent. w/v Na_2CO_3. This stock solution was prepared once every 2 weeks and was stored in the dark at 4° C. Before use, 0·01 ml. of stock solution was diluted with 1·5 ml. of plasma to give a concentration of $1{\cdot}6 \times 10^{-4}$ g T_3/l. in the final medium (Fell & Mellanby, 1956). An equivalent amount of sodium carbonate solution was added to the control medium.

The final volume of medium in each watch-glass ranged from 0·6 to 1·0 ml. according to the size and number of the explants. Not more than three rudiments from one side of an embryo were cultivated in each dish containing T_3, and the corresponding rudiments from the other side were grown in a similar dish without the hormone. The explants were transferred to fresh medium every other day and the experiments ended after 8 days.

Measurement of the rudiments

Length. The lengths of the growing rudiments were calculated from daily camera lucida drawings of the explants (Fell & Mellanby, 1952).

Wet weight. Excess moisture was removed from the explants at the end of the culture period by rolling them once on hard filter-paper (Whatman No. 50); they were then weighed in sealed capillary tubes.

Total nitrogen content. Groups of four rudiments from 6-day-old embryos were digested by the method of Ma & Zuazaga (1942). Total nitrogen was estimated by the micro-Kjeldahl method (Markham, 1942); the ammonia was collected in a 2 per cent. w/v solution of boric acid and titrated with 0·005 N HCl in the presence of Sher's (1955) indicator.

Estimation of response to T_3

The effect of T_3 on the growth in length and wet weight of the whole rudiments was estimated in the following ways:

(i) The increments of growth in length of the control and treated rudiments were measured every 2 days and the significance of the difference tested by Student's *t*-test for paired samples.

(ii) The response at the end of the culture period was expressed as the ratio of the final lengths, or wet weights, of the treated and control rudiments. Statistical comparison of the response of different bones was made with logarithms of the measurements, i.e. response $= \log_{10}T - \log_{10}C$, where T = length or wet weight of the treated rudiment and C = length or wet weight of the paired control. The standard error was calculated from the difference in response of any two bones within the same embryo in order to exclude the variation between embryos.

THE EFFECT OF DEVELOPMENTAL STAGE ON THE RESPONSE OF THE TIBIA AND RADIUS TO T_3

Fell & Mellanby (1955, 1956) found that T_4 stimulated the initial growth in length of humeri in explanted limb blastemata, whereas the growth of rudiments from older embryos was retarded by both T_4 and T_3. The growth of the radii from these older embryos was stimulated by both hormones, but when the embryos were divided into two groups on the basis of stage of development, Fell & Mellanby found that the growth of the radii of the younger group was stimulated by T_3 while the growth of the bones in the older group was unaffected. The conclusion that the differential response was influenced by the stage of development at which the rudiments were exposed to the hormone was supported by the response of the other long bones which were tested. These results suggested that the developmental stage may be the most important factor in eliciting a differential response from the different bones of the same embryo, since the rudiments are at different stages of differentiation at any given time. In the experiments described in this section, this possibility was tested by comparing the response of the tibia and radius to T_3 at various stages of development; these two rudiments were chosen because of all the long bones from the 6-day-old embryo they show the most widely divergent response.

Experimental

The embryos from 5- to 8-day-old eggs were divided into groups, roughly according to age, and precisely according to the initial lengths of the bone rudiments and stage of development when viewed through the dissecting microscope (Table 1). Division of the rudiments into groups on the basis of morphological appearance agreed with their subsequent grouping according to initial length, and there was no overlap of initial length between the groups. The radii of any group were at least as mature as the tibiae of the preceding group. No results for group A radii are given, since both treated and control explants developed merely as nodules of small-celled cartilage.

TABLE 1

Division of embryos into groups on the basis of the initial stage of differentiation of the tibia and radius

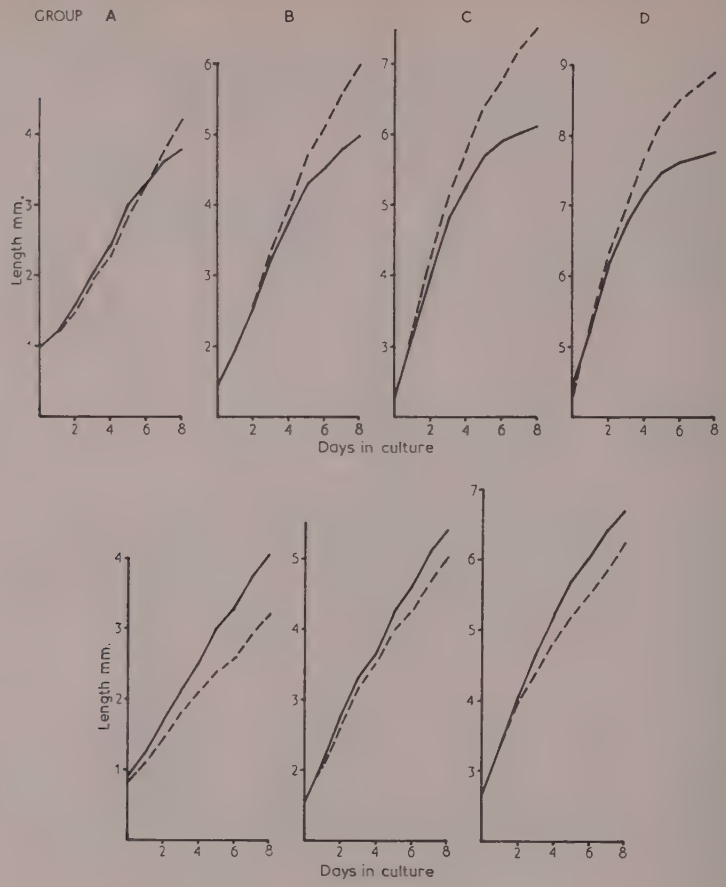
Group	No. of embryos	Age	Tibia		Radius	
			Morphology	Length	Morphology	Length
		(days)		(mm.)		(mm.)
A	9	5–5½	Procartilage mass, ends indistinct	0·75–1·2		
B	12	5½–6	Distinct rudiment, limited by perichondrium round shaft, ± hypertrophy	1·27–1·7	More advanced than tibia (A), but less than (B)	0·6–1·0
C	12	6–7	Hypertrophy usually distinct in centre of shaft; band of yellowish osteoid tissue round shaft. Epiphyses distinct	1·7–3·2	As tibia (B–C)	1·1–2·1
D	10	8	Calcification begun in osteoid which now extends to flattened-cell zone	3·4–5·8	Shaft is clearly hypertrophic but no sign of calcification in osteoid	2·1–2·6

Results

There was a slight stimulation of the growth in length of the group A tibiae treated with T_3 during the first 2 days in culture (Text-fig. 1). This increase was statistically significant at the 1 per cent. level, but was slight compared with that of the group B radii with which the group A tibiae were histologically comparable (Text-fig. 1). The hypertrophic areas of the T_3-treated tibiae were slightly more prominent and extensive in the living cultures after the first 2 days, and the transitory increase of growth in length may be the result of the slightly earlier onset of hypertrophy, as was found in the young humeri treated with T_4 (Fell & Mellanby, 1955). During the last 2 days in culture the T_3-treated tibiae in group A showed the retardation in growth characteristic of tibiae from older groups.

T_3 also stimulated the growth in length of radii in groups C and D, but the effect was less than on radii in group B (Text-fig. 1). The only suggestion of retardation was in radii of group D during the last 2 days of culture, but this was not statistically significant.

Thus tibia and radius at comparable histogenetic stages respond differentially to T_3. The precocious onset of hypertrophy produced by T_3 in the youngest groups of both bones is probably responsible for the transitory increase in growth in length of the tibia, and the much greater stimulation of the radius as compared with the radii of the older groups.



TEXT-FIG. 1. Response of the tibia and radius at different developmental stages. *Upper row*: tibia; *lower row*: radius. The dotted lines represent the length of the control rudiments, the full lines the length of the equivalent rudiments treated with T_3. The growth of the treated tibiae in all groups is retarded except for a transitory stimulation of the bones in group A. In contrast the growth of the radius is stimulated.

THE EFFECT OF RUDIMENT SIZE ON THE DIFFERENTIAL RESPONSE

In organ culture the supply of food to the explant is limited by the periodic replacement of the medium and by the rate of diffusion of materials through the medium. The conditions in such a culture may be more favourable for the growth of a small rudiment with a relatively large surface–volume ratio like the radius, than for the comparatively bulky tibia. The utilization of amino-acids and glucose per unit wet weight of bone rudiment is greater for the radius than for the tibia (Lawson & Lucy, 1961; and Lawson, unpublished observation).

It is possible that the retardation in the growth of tibiae treated with T_3 is due to the limitations in food supply to the larger bone.

The response of the tibia was therefore compared with the responses of five small bones: third metatarsus, radius, ulna, third metacarpus, and fourth metacarpus.

Experimental

Rudiments from twelve 6-day-old embryos were cultured, and the initial lengths of the bones were used as an indication of their initial size. The total nitrogen of the rudiments of other embryos from the same batch were determined for comparison. The response to T_3 was measured at the end of the culture period.

Results

The responses of the five small bones varied from severe retardation of the growth of the third metatarsus to marked stimulation of the fourth metacarpus (Table 2); the distinctness of the responses of the different bones is indicated in Table 3. When the bones were arranged in a series according to their response to T_3, the order was the same whether the response was measured on the final length or on the wet weight of the whole rudiments. Table 2 shows that the predominant response in this series was an increase in wet weight and retardation of growth in length; the epiphyses of all the treated rudiments were much enlarged, and in the ulna and third metacarpus this more than compensated for any loss in weight from the slight reduction in length.

TABLE 2

Final response of different bones from the same embryos to T_3

	Response		*Initial length*	*Initial total nitrogen*	*Final control wet weight*
	Length	*Wet weight*	(mm.)	(μg.)	(mg.)
Tibia . . .	82	74	2·2	8·4	6·46
Metatarsus$_3$ . .	84	82	1·4	2·4	2·39
Ulna . . .	91	108	1·7	3·3	3·54
Metacarpus$_3$ . .	96	119	1·1	2·3	1·48
Radius . . .	102	170	1·4	2·6	1·06
Metacarpus$_4$ . .	112	190	0·9	1·6	0·37

No. of embryos used = 12. The response quoted is the treated specimens as a percentage of the control specimens.

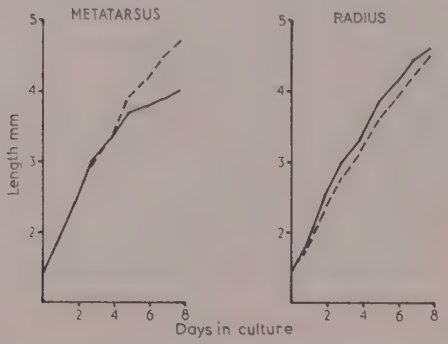
The degree of response was not associated with the initial size of the different bones (Table 2). The absence of association was particularly evident in the metatarsus and radius, which were of the same initial size but showed strikingly different growth-responses (Text-fig. 2); the retardation of the growth of the metatarsus was comparable with that of the tibia (Table 3).

TABLE 3

The statistical significance of the differential response to T_3

Difference between:	*Length* ($n = 11$)			*Wet weight* ($n = 8$)		
	*Mean difference in response**	*Standard error of the mean difference*	*P*	*Mean difference in response**	*Standard error of the mean difference*	*P*
Tibia and metatarsus$_3$	0·020	0·007	< 0.05	0·029	0·017	> 0.05
Metatarsus$_3$ and ulna	0·023	0·007	< 0.01	0·107	0·018	< 0.001
Ulna and metacarpus$_3$	0·027	0·009	< 0.05	0·036	0·007	< 0.01
Metacarpus$_3$ and radius	0·033	0·010	< 0.01	0·144	0·037	< 0.01
Radius and metacarpus$_4$	0·042	0·013	< 0.05	0·039	0·038	> 0.05
Metatarsus$_3$ and radius	0·075	0·009	< 0.001	0·283	0·051	< 0.001

* Response calculated as $\log_{10} T_3 - \log_{10}$ control.



TEXT-FIG. 2. Differential response of the third metatarsus (*left*) and the radius (*right*) to T_3. Dotted lines represent the length of control rudiments, the full lines show the length of the equivalent rudiments treated with T_3. The two bones were of similar size and at the same histogenetic stage when initially exposed to the hormone.

A series of control bones arranged in order of final wet weight corresponded with the series of bones arranged in order of their response to T_3 (Table 2), except that the reduction of the growth in length of the metatarsus was significantly greater than that of the larger ulna; indeed, the final wet weight of the treated ulna was greater than that of the control, whereas the final wet weight of the treated metatarsus was much less than that of its control (Tables 2, 3). The difference between the order of the bones arranged according to final size and when arranged according to initial size presumably reflects differences in growth rate; the connexion of the differential growth rate with the differential response to T_3 will be examined in a later paper.

In this experiment different bones were taken from the same 6-day-old embryos and were therefore at varying stages of development. At this age the developmental stages of the rudiments fall in the same order as their initial sizes. Thus the tibia is the largest and the most advanced, the fourth metacarpus is the smallest and least developed, while the metatarsus and the radius are the same size and at similar stages; any correlation between response and initial bone-size would therefore be enhanced. Although the experiment clearly demonstrated that rudiments of the same size respond differently to T_3, it cannot be concluded from the results that the response is not modified at any stage by the size of the treated rudiment.

CONCLUSIONS

It may therefore be concluded that the differential growth response to T_3 *in vitro* is influenced but not determined by the developmental stage of the rudiments, and that differences in the initial size of different rudiments do not determine the differential response *in vitro*.

SUMMARY

1. The cartilaginous limb-bone rudiments of the 6-day-old embryonic chick show a differential growth response to treatment with triiodothyronine (T_3) during 8 days' culture *in vitro*. The growth in length and wet weight of the tibia is reduced by $1{\cdot}6\times10^{-4}$g. T_3/l. of medium, but the growth of the radius is increased.

2. Tibia and radius explanted at comparable histogenetic stages respond differently to T_3, but the responses are slightly modified by the stage of development at which the rudiments are exposed to the hormone: tibiae from 5- to 5½-day-old embryos show a transitory stimulation of growth in length, followed by retardation, while increase in the growth in length of radii from 7- and 8-day-old embryos is less than that of radii from 6-day-old embryos.

3. Five small limb-bone rudiments of a similar size (third metatarsus, radius, ulna, third metacarpus, and fourth metacarpus) respond differentially to T_3. It is therefore unlikely that the differential growth-response of the tibia and radius is a result of the limited nutritional conditions *in vitro*.

RÉSUMÉ

La réaction différentielle de croissance dans les rudiments d'os de membres de l'embryon de Poulet à la triiodothyronine in vitro

I. *Stades du développement et taille des organes*

1. Les rudiments cartilagineux des os des membres du poulet embryonnaire de 6 jours montrent une réaction différentielle de croissance au traitement à la triiodothyronine (T_3) pendant 8 jours de culture *in vitro*. La croissance en

longueur et en poids du tibia est réduite par $1{\cdot}6 \times 10^{-4}$g. T_3/l. du milieu, mais la croissance du radius est augmentée.

2. Le tibia et le radius explantés à des stades comparables de l'histogenèse ont des réactions différentes à T_3, mais la réponse est modifiée légèrement par le stade de développement auquel les rudiments sont exposés à l'hormone: des tibias d'embryons de 5 à $5\frac{1}{2}$ jours montrent une stimulation transitoire de la croissance en longueur, suivie d'un retard, tandis que l'augmentation de la croissance en longueur des radius d'embryons de 7 et 8 jours est moindre que celle des radius d'embryons de 6 jours.

3. Cinq petits rudiments d'articles osseux de taille voisine (le 3e métatarsien, le radius, le cubitus, le 3e métacarpien et le 4e métacarpien) ont des réactions différentes à T_3. Il est donc peu vraisemblable que la réaction différentielle de croissance du tibia et du radius soit le résultat des conditions restrictives de nutrition réalisées *in vitro*.

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The Serum Proteins of the Rat During Development

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INTRODUCTION

THE morphological changes which occur during late embryonic and early postnatal life in common laboratory animals are, with one set of exceptions, now well understood. The exception relates to those changes which require the use of physiological, biophysical, or biochemical techniques. Outstanding among these are the changes in the relative concentrations of the serum proteins (Kekwick, 1959). Only for the chicken (Moore, Shen, & Alexander, 1945; Marshall & Deutsch, 1950; Heim & Schechtman, 1954; Weller & Schechtman, 1957) has a reasonably complete picture been built up.

In the case of the changes in the relative concentrations of the serum proteins of the late embryonic and early postnatal rat, the various investigations, including the more extensive ones of Jameson, Alvarez-Tostado, & Lew (1948), Shmerling & Uspenskaya (1955), and Gurvich & Karsaevskaya (1956), leave a confusing and contradictory picture due to the use of widely divergent methods and the limited number of stages investigated.

In view of this, a systematic survey of the serum proteins of the rat, from the earliest suitable developmental stage to the immediate postnatal stages, was undertaken with the aid of a highly standardized technique. Sera of adult male and female rats were also examined as controls and for purposes of comparison.

MATERIALS AND METHODS

All animals used were Sprague–Dawley rats, obtained from the Holtzman Company, or their descendants, bred in this laboratory. The animals were between 90 and 200 days old when mated. They were maintained on an *ad libitum* diet of Purina rat chow and water supplemented with occasional feedings of raw pork-liver, hard-boiled egg-slices, or slices of orange. The date of insemination was determined by the finding of spermatozoa in daily vaginal smears. Pregnancy was assumed to have begun at about 7 a.m. on the morning on which spermatozoa were found in the smear. In order to maintain proper timing, all bleeding was done between 7 and 8 a.m. Delivery was assumed to have occurred, and generally did occur, early during the 23rd day of pregnancy. This day is designated hereafter as the first day post-partum.

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Blood was obtained in one of three ways. In the procedure used with most foetuses, the mother was anesthesized with ether, her abdominal and uterine cavities opened, and the foetuses successively removed from their membranes. A small, canoe-shaped piece of lucite was slipped under the umbilical cord. After drying off the outside of the cord, one or more vessels within it were nicked and the blood collected in a siliconed pipette as it escaped into the plastic boat. In the cases of some of the older foetuses and of the new-born rats, a deep dorsal incision was made into the neck, care being taken to avoid cutting into the trachea or oesophagus. The first drop of blood to appear was usually discarded and the next few drops collected directly into a siliconed centrifuge tube. The blood from at least four foetuses or neonatal animals from the same litter was pooled. The immature and adult animals were bled by direct heart-puncture under light ether anesthesia. In all cases, the blood was allowed to clot for approximately 4 hours in siliconed centrifuge tubes at about 4° C. After brief centrifugation, the supernatant serum was removed and its refractive index determined with white light in an Abbe-type refractometer. No dilutions were made.

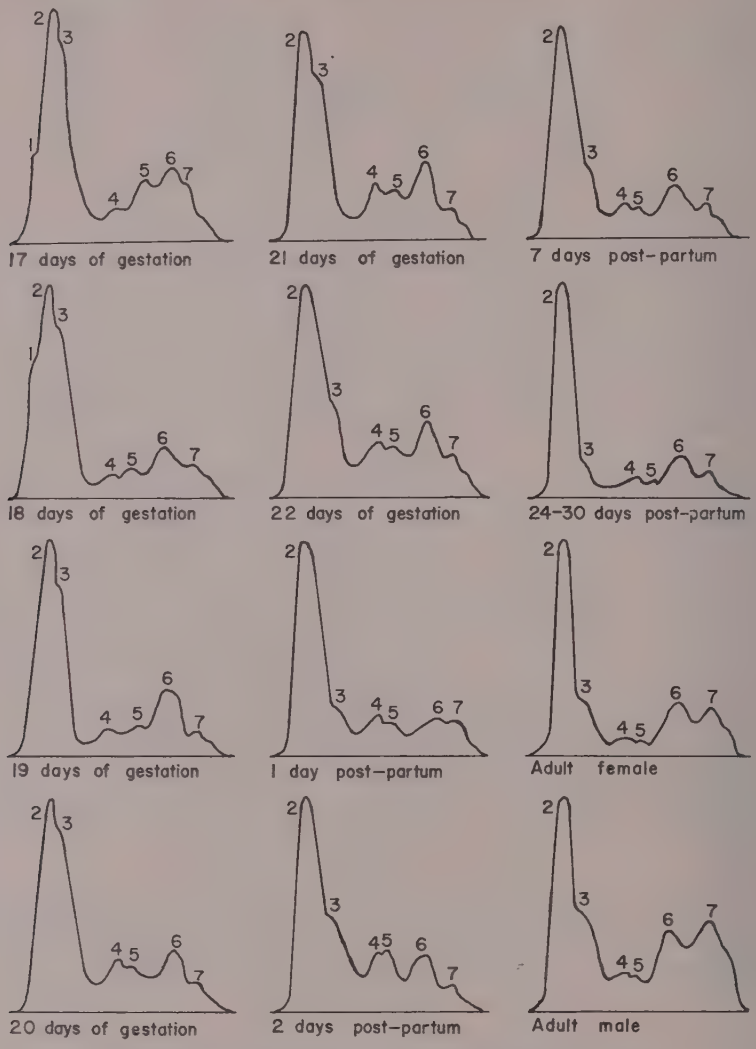
Paper electrophoresis was carried out in accordance with the instructions (Publication RIM-5, 1957) for, and utilizing the equipment of, the Spinco model R system. The buffer was barbital (5,5-di-ethylbarbituric acid) 2·76 g.; barbital sodium (2-sodium, 5,5-di-ethylbarbituric acid) 15·40 g.; distilled water to make one litre. This buffer is 0·075 M in the sodium salt and has a pH of 8·6. 0·010 ml. of adult serum was applied to each paper strip. The volume of serum from the earlier stages applied to each strip was adjusted so that the amount of protein, as estimated from the refractive index, was approximately equal to that in 0·010 ml. of adult protein. This precaution proved necessary because the resultant pattern is somewhat dependent on the amount of protein applied. A potential gradient of 2·6 v./cm., resulting in a current flow of 0·31 ma. per strip, was applied under constant current conditions for 19 hours. After staining with bromphenol blue, photometric evaluation was performed with the Spinco Analytrol. Components were delimited by dropping verticals (Tiselius & Kabat, 1939) and the relative concentrations were calculated from the ratio of the area under each section of the curve to that under the total curve.

RESULTS

Typical patterns obtained from the sera of the various developmental stages examined are shown in Text-fig. 1. The leading component visible in the patterns of animals of 17 and 18 days of gestation has an anodic mobility well within the range shown by adult albumin. An asymmetry on the trailing edge of the gamma globulin component is evident at all stages. The leading edge of the beta globulin fraction shows considerable asymmetry in the neonatal and immature stages. No significant changes in the mobilities of the components were detected.

The percentage composition of the serum proteins at the various develop-

mental stages examined is given in Table 1 and the changes in these percentages during development are shown in Text-fig. 2. Albumin constitutes the largest



TEXT-FIG. 1. Typical patterns of sera obtained at various stages of development. Component 1: fast albumin. Component 2: albumin. Component 3: alpha-1 globulin. Component 4: alpha-2 globulin. Component 5: alpha-3 globulin. Component 6: beta globulin. Component 7: gamma globulin.

single component at all stages. The fast albumin fraction loses its identity as a component distinct from the main mass of albumin between the 18th and 19th days of gestation. The changes in the relative concentrations of albumin and

alpha-1 globulin are generally opposite to each other. Gamma globulin does not begin to approach adult level until after at least one month of postnatal life.

TABLE 1

Relative concentration of the serum protein fractions

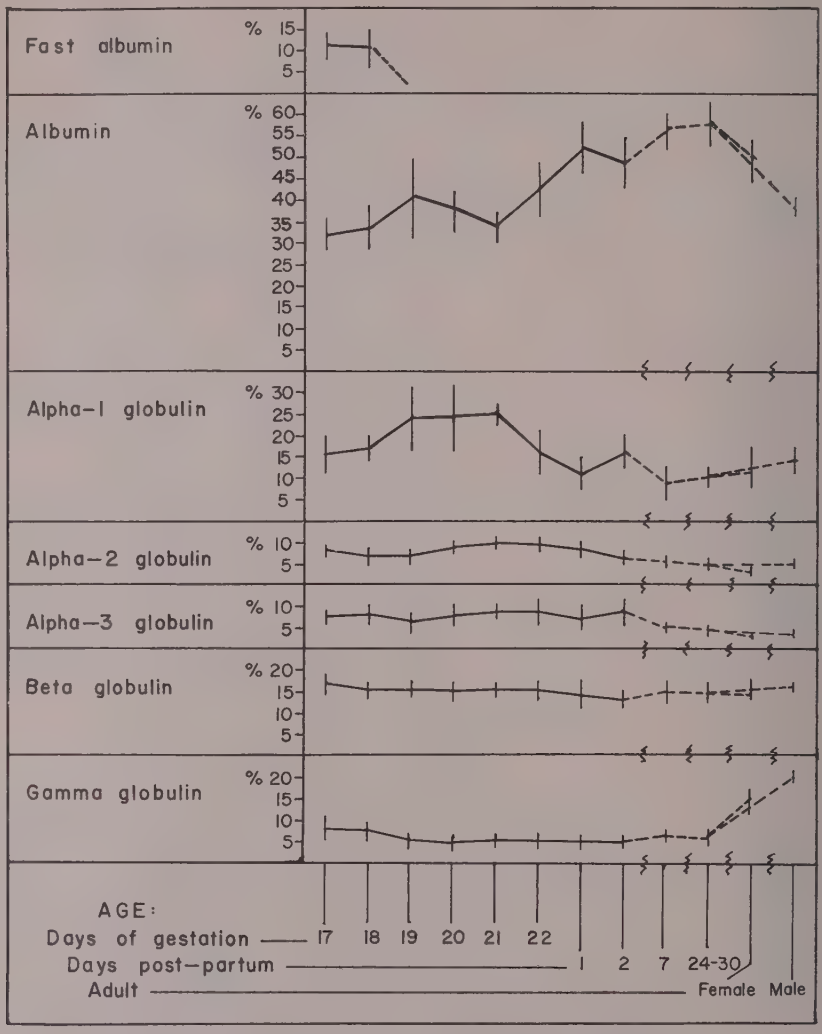
Age	No. of samples	No. of analysis	Relative concentration in percent						
			Fast albumin	Albumin	Alpha-1 globulin	Alpha-2 globulin	Alpha-3 globulin	Beta globulin	Gamma globulin
17 days' gestation	10	35	11·3*	31·7	15·9	8·3	7·7	16·9	8·2
			±2·9	±4·3	±4·5	±1·2	±1·3	±2·8	±3·1
18 days' gestation	9	36	11·0	33·6	17·1	7·0	8·5	15·4	7·4
			±4·3	±5·2	±2·3	±2·2	±2·2	±2·0	±2·1
19 days' gestation	10	40		40·8	24·2	7·1	6·8	15·7	5·4
				·9·4	·7·8	·1·6	·2·2	·2·1	·1·7
20 days' gestation	10	39		37·8	24·4	9·1	8·3	15·2	5·2
				±5·0	±7·8	±1·3	±3·0	±1·9	±1·3
21 days' gestation	9	35		33·7	25·0	10·0	9·2	16·2	5·9
				±3·1	±2·1	±1·1	±1·7	±1·8	±1·1
22 days' gestation	13	53		42·6	16·7	9·8	9·4	15·8	5·7
				±6·6	±5·5	±1·7	±3·4	±2·0	±1·8
1 day post-partum	12	60		52·4	11·5	8·5	7·7	14·6	5·3
				±6·4	±3·6	±1·6	±3·3	±3·5	±1·8
2 days post-partum	13	56		48·7	16·5	6·7	9·2	13·5	5·4
				±6·6	±4·2	±1·8	±3·5	±2·3	±1·1
7 days post-partum	14	71		56·4	9·2	6·3	5·6	15·5	7·0
				±4·5	±3·9	±1·2	±1·0	±2·7	±1·3
24–30 days post-partum	7	28		58·1	10·6	5·6	4·5	15·1	6·1
				±5·4	±2·3	±1·2	±0·9	±2·3	±1·3
Adult female	19	71		49·8	12·9	3·7	3·4	15·5	14·7
				±4·7	±5·4	±0·6	±0·6	±2·5	±3·3
Adult male	7	28		38·8	14·4	5·6	4·1	16·7	20·4
				±2·5	±3·2	±0·9	±0·5	±0·8	±1·5

* Percentage ± standard deviation.

DISCUSSION

Gurvich & Karsaevskaya (1956) report the presence of a pre-albumin component in the sera of rat embryos weighing from 0·43 to 0·72 g. According to the data of Stotsenburg (1915), such animals would be between about $16\frac{1}{2}$ and $17\frac{1}{2}$ days of gestation. The average weight for rats of 17 days of gestation was found to be 0·74±0·05 g. in the present experiment, thus confirming the above age estimates. It is in this same age-range, and only in this age-range, that the fast albumin component was detected in the present work. The relative concentration of the pre-albumin listed by Gurvich & Karsaevskaya (1956) ranges from 16·00 to 21·0 per cent., whereas the largest average value measured in the present work was 11·3±2·9 per cent. It is likely, therefore, that the pre-albumin fraction of these workers and the fast albumin component found in the present work include, at least in part, the same protein groups. The term 'pre-albumin' refers to a protein fraction having a greater anodic mobility at a pH above its isoelectric point than does the family of proteins designated as albumin. Such components are found, for example, in the sera of chicken embryos and laying hens (Heim

& Schechtman, 1954). In the present case, however, the leading edge of the component in question does not exhibit a greater mobility than that of the leading



TEXT-FIG. 2. Average percentage composition of electrophoretic components in rat serum from the 17th day of incubation to the mature adult. The short vertical lines indicate the range of plus or minus one standard deviation. The dashed lines connect points plotted on an interrupted time scale and hence their slope does not represent a rate of change.

edge of the albumin fraction of the adult or of later foetal stages. Secondly, it may be seen from Text-fig. 2 that the disappearance of the fast albumin on the 19th day of gestation is accompanied by a nearly proportional increase in the

relative concentration of the albumin. This suggests that the material previously recognized as fast albumin does not disappear at all but that it becomes merged with the main bulk of the albumin group. If the fast albumin material had disappeared, all remaining components would be expected to show a compensatory increase in relative concentration, proportional to the fraction each constitutes of the total protein concentration. Actually, only two of the six components show any significant increase between the 18th and 19th days of development. Supporting evidence for the hypothesis that the fast albumin fraction becomes merged with the main body of albumin comes from the great variability in the relative concentration of albumin in the serum of the animals at the 19th day of gestation as shown by the high standard deviation value (Table 1). The loss of distinction between the fast albumin and the albumin may be due to the appearance of a relatively small amount of a new albumin sub-fraction having a mobility intermediate between those of the fast albumin and the peak of the main albumin mass. The appearance of new serum proteins during the course of rat ontogeny has been postulated on immunochemical grounds by Gurvich & Karsaevskaya (1956).

An asymmetry was found in the trailing region of the gamma-globulin fraction during all stages of development, including the adult. An asymmetry very similar in appearance and position was also found by Gurvich & Karsaevskaya (1956), but only in sera from perinatal animals. These workers have designated one component of this complex as eta globulin and consider it, on immunochemical grounds, to be a distinct protein family. Since in the present work, however, this component or asymmetry appears at all stages of development and always at or near the point of initial application of the serum, it is suggested that the asymmetry is an artifact due to adsorbed proteins. Gurvich & Karsaevskaya (1956) have recognized that, in their immunoelectrophoretic procedure, confusion can arise between an immunological precipitate and an adsorbed protein. Contrary to the findings of Shmerling & Uspenskaya (1955), gamma globulin could be demonstrated in the sera from all stages examined.

Shmerling & Uspenskaya (1955) report the presence, in embryos weighing 2 g. or more and in rats one day after birth, of an alpha-2 and an alpha-4 globulin which more or less completely replace the alpha-1 and alpha-3 globulins found in other developmental stages. They believe the former two components to be analogous to foetuin (Pedersen, 1947). Some support is lent to this view by the finding of a considerable degree of asymmetry in the beta-globulin peak of sera from perinatal and juvenile animals. The observation of these authors that the sera of suckling young are cloudy is confirmed, although occasionally nearly clear sera were obtained. Since optical clarity is not a prerequisite for paper electrophoresis, as it is for the classical Tiselius technique, no defatting process was used. The present work also confirms the view of these workers that, on the basis of their mobilities, the fractions of the same denomination in embryonic and adult sera are electrophoretically identical.

The observation that the relative concentration of gamma globulin of immature rats remains for an extended period below the levels found in the adult is in accord with the finding of Halliday & Kekwick (1957).

That the alpha-globulin fraction or series of fractions was found in the present work but not in that of Jameson, Alvarez-Tostado, & Lew (1948) may be due to the use of very different buffer systems.

The comparatively constant relative concentration of alpha-2, alpha-3, beta, and gamma globulins during intra-uterine existence may indicate that the embryonic pools of these components are in rather free communication with the corresponding, larger, maternal pools. Should this be the case, we would be presented with the physiologically interesting situation in which the internal environment of one organism, the foetus, changes in response to the demands placed upon, and the response pattern of, another organism, namely, the maternal one. The relations between the serum proteins of mother and foetus and the possible selective action of the placenta on the exchange of serum proteins is presently under study.

SUMMARY

1. The sera of rats at various stages of development from the 17th day of gestation to adulthood were examined by paper electrophoresis.

2. A component distinct from, and of higher mobility than, the bulk of the albumin was found at the 17th and 18th days of development. However, the mobility of this component is well within the limits of mobility of adult albumin.

3. Evidence supporting the existence of a component in the beta-globulin region specific to the perinatal animal is presented.

4. Gamma globulin and an asymmetry associated with it were demonstrable in all stages examined. The possible relation of this asymmetry to eta globulin is discussed.

5. Problems concerning the physiology of the foetus arising from the changes in the relative concentrations of the serum proteins are pointed out.

RÉSUMÉ

Les protéines du sérum du Rat pendant le développement

1. Les sérums de rats ont été étudiés à différents stades du développement à partir du 17^{e} jour de la gestation jusqu'à l'âge adulte par la méthode de l'électrophorèse sur papier.

2. Un composant distinct et d'une plus grande mobilité que la plus grande partie de l'albumine à ce stade a été mis en évidence aux 17^{e} et 18^{e} jours du développement. Cependant, la mobilité de ce composant est bien dans les limites de mobilité de l'albumine adulte.

3. Il y a de sérieuses raisons d'admettre l'existence d'un composant spécifique situé dans la région de la béta-globuline, aux environs de la naissance.

4. A tous les stades étudiés, on a démontré l'existence de gamma-globuline et de l'asymétrie qui lui est associée. La possibilité d'une relation de cette asymétrie avec l'eta globuline est discutée.

5. L'accent est mis sur les problèmes concernant la physiologie du fœtus en rapport avec les changements dans les concentrations relatives des protéines du sérum.

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La Métachromasie au bleu de toluidine dans l'œuf d'*Artemia salina*

I. Aspect, distribution et origine des grains chromotropes M

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AVEC UNE PLANCHE

INTRODUCTION

Le plasme périnucléaire de l'œuf d'*Artemia salina* en segmentation présente une composition complexe. On y rencontre des granules et des éléments structurés d'aspect et de composition chimique très divers. Il est bourré d'un semis de très fins granules riches en ribonucléoprotéines et contenant également des protéines sulfhydrilées. Il se pourrait que leur aspect granuleux soit un artéfact de fixation et qu'il s'agisse plutôt de constituants du réticulum endoplasmique. Ils sont mêlés à des granules plus épais qui contiennent du glycogène et des protéines basiques (J. Fautrez & N. Fautrez-Firlefyn, 1959). Le plasme périnucléaire est en outre riche en mitochondries. Ces dernières sont facilement mises en évidence par la technique de Baker pour les phospholipines. Leur nature mitochondriale a d'ailleurs été confirmée par le fait qu'elles apparaissent en noir au microscope à contraste de phase et par leur aspect au microscope électronique. Disposées, au cours de l'interphase, en un gradient décroissant à partir de la membrane nucléaire, elles effectuent des déplacements au cours de la mitose, que l'une d'entre nous a décrit ailleurs (N. Fautrez-Firlefyn, 1960). Éparses parmi ces éléments, on rencontre de larges sphères hyalines que nous n'avons jamais pu colorer ni par des méthodes histologiques ni par des réactions cytochimiques. Ces sphères sont expulsées par le noyau, surtout au début de la prophase. Restent enfin des grains intensivement chromotropes, que l'on met aisément en évidence au moyen du bleu de toluidine. Ce sont ces derniers, qui nous ont longuement intrigués, que nous voudrions étudier ici de plus près.

Ces granules ne sont pas sans rappeler par certains caractères les granules chromotropes α et β, étudiés par A. Dalcq, J. Pasteels et par leurs collaborateurs dans les œufs de diverses espèces marines et de mammifères (cf. Dalcq, 1960, pp. 332 sqq.). Insistons cependant sur le fait que l'école bruxelloise a obtenu la métachromasie au bleu de toluidine *in vivo*. Sur notre matériel opaque, l'ob-

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servation sur le vivant est impossible sauf pour des éléments corticaux sur lesquels nous reviendrons ailleurs. Les grains que nous étudierons ici sont chromotropes dans les œufs fixés. Inversement il ne semble pas nettement établi si les granules α et β des auteurs bruxellois restent chromotropes après fixation.

MATÉRIEL ET TECHNIQUE

Nous utilisons dans nos travaux une *Artemia salina* amphimixique diploïde d'origine californienne. A partir d'œufs enkystés que l'on trouve dans le commerce comme nourriture pour poissons d'aquarium, nous entretenons au laboratoire des cultures dans une eau salée à 30 pour mille.

Des femelles gravides sont fixées après décapitation afin de s'assurer d'une pénétration rapide du fixateur et coupées en série. Les coupes sont colorées au bleu de toluidine. Dans ces conditions la métachromasie obtenue ne dépend pas uniquement de la concentration et du pH de la solution colorée utilisée, mais également du fixateur employé. Pour le moment nous utiliserons les conditions optima pour l'apparition de la métachromasie, afin d'étudier plus spécialement l'aspect, la distribution et l'origine des granules chromotropes. Les animaux sont fixés à l'alcool-formol acétique (75+20+5) ou à l'alcool-formol salé (alcool 70 p.+formol salé de Baker 30 p.). La solution colorée est une solution aqueuse de bleu de toluidine à 1/5.000 à pH 5 à 5·8. Notons en outre que la technique de la coloration même a une grande influence sur le résultat. On obtient en effet une différenciation importante lors du passage par les alcools. C'est ainsi que le passage de l'eau dans les alcools à 70° et à 90° fait disparaître la métachromasie β (pourpre) de Michaelis (1947), et non la métachromasie γ (rouge). Pour obtenir des résultats comparables on doit porter les préparations sur une même lame porte-objet ou sur des lames traitées dos à dos. Comme les faits que nous décrivons ici ne peuvent être recueillis que dans un matériel très abondant, rassemblé au hasard de fixations différentes, cette méthode n'est pas toujours utilisable. Dès lors nous avons essayé d'éviter le passage par les alcools dilués; au sortir du bleu de toluidine les coupes sont essorées et plongées immédiatement dans l'alcool absolu. Dans ces conditions une étude comparative devient possible.

OBSERVATIONS PERSONNELLES

1. *L'intercinèse au cours des premiers stades de la segmentation*

Nous examinerons d'abord les granules chromotropes M au cours des premiers stades de la segmentation; c'est à ce moment qu'on les retrouve le plus aisément. Au moment de l'intercinèse les granules M sont surtout épars dans le plasme périnucléaire. A partir du stade II la partie de ce plasme dirigée vers le centre du germe en est moins richement pourvue (Planche, fig. 9). Ces granules se présentent comme des structures complexes, dont la partie principale est une flaque irrégulièrement lancéolée, en forme de virgule ou de gros grain et présentant une métachromasie très intense. Accolés à cette flaque, on trouve un ou

plusieurs petits granules denses d'un bleu très sombre. Ces structures tranchent nettement sur le fond du plasme périnucléaire qui est légèrement pourpre (métachromasie β due au RNA).

Une étude plus approfondie permet en outre de trouver au sein du vitellus des éléments qui semblent à l'origine des grains chromotropes du plasme périnucléaire. Alors que la plupart des grains de vitellus se colorent de manière homogène et en une teinte orthochrome au bleu de toluidine, un assez grand nombre d'entre eux présente de petites vacuoles de forme ovoïde, non colorables et fortement réfringents (Planche, fig. 1). C'est au sein de ces vacuoles que s'accumule une substance, qui dès le début présente une métachromasie γ très prononcée, tandis que la plaquette vitelline diminue habituellement de taille tout en se colorant plus intensivement et en virant progressivement au bleu violacé (Planche, fig. 2). Puis les vacuoles confluent en deux ou trois grains rouges, tandis que les dimensions de la gaine violacée (métachromasie β) continuent à diminuer (Planche, fig. 3). Entre ces éléments que nous appellerons 'granules M jeunes' et les granules M mûrs du plasme périnucléaire (Planche, fig. 4) on peut trouver toutes les transitions. En étudiant la distribution topographique de ces diverses structures, on a d'ailleurs l'impression que cette évolution s'effectue en direction du plasme périnucléaire et qu'en 'mûrissant' les granules M migrent en direction du noyau.

2. *Évolution des grains chromotropes au cours de la maturation et de la segmentation*

Dans l'oöcyte ovarien en accroissement cytoplasmique on ne trouve jamais de grains chromotropes. Ils font leur apparition en même temps que le vitellus. Lorsque les premières plaquettes vitellines se forment dans la partie centrale du germe, on peut observer de-ci de-là dans cet amas un petit granule chromotrope. Lorsque la vitellogénèse touche à sa fin, on rencontre ces éléments encore peu nombreux et généralement de petite taille irrégulièrement distribués dans tout le corps cellulaire. Il s'agit surtout d'éléments chromotropes 'jeunes': une coque ovoïde bleue ou violacée, contenant plusieurs granules rouges.

Il est à remarquer toutefois que dans les cellules vitellogènes, qui délimitent la cavité utérine, aucun granule M n'a jamais été mis en évidence.

Lorsque les œufs poursuivent leur maturation d'abord dans les sacs latéraux des oviductes, ensuite dans l'utérus, le nombre des granules chromotropes 'jeunes' augmente assez rapidement; leur distribution topographique reste la même jusqu'au moment de la copulation des pronucléi. Aucune concentration ne s'observe autour des mitoses de maturation (Planche, fig. 5). Ni le pronucléus mâle (Planche, fig. 6), ni l'aster spermatique (Planche, fig. 7) ne semble attirer spécialement les grains chromotropes.

En séparant des femelles très jeunes des mâles, il est possible d'obtenir des animaux mûrs mais vierges. Dans ces conditions les œufs mûrs descendent dans les sacs latéraux des gonoductes. C'est à cet endroit que, comme normalement

d'ailleurs, on les retrouve en métaphase de la première division de maturation. Comme les œufs ne sont pas fécondés, la maturation ne va pas plus loin, et les germes ne quittent pas le sac latéral vers l'utérus. De nouveaux oöcytes entrent cependant en vitellogénèse au niveau de l'ovaire: après quelque temps on obtient des femelles dont les sacs latéraux et les ovaires sont bourrés d'œufs, alors que l'utérus est vide. Dans ces conditions on constate également que les premiers grains chromotropes apparaissent en même temps que les premières plaquettes vitellines et que dans l'œuf en vitellogénèse descendu dans les sacs latéraux leur nombre accroît considérablement; il dépasse singulièrement celui que l'on trouve au même stade dans des femelles fécondées. Tout se passe comme si la production de grains chromotropes y continuait indépendamment du fait que la maturation y est bloquée au stade de la métaphase de la division réductionnelle.

Dans les œufs fécondés, un changement de distribution s'opère dès le début de la prophase de la première division de segmentation. Les granules M chromotropes 'mûrs' ont tendance à venir se grouper dans l'énorme aster qui se développe au contact des deux pronucléi accolés (Planche, fig. 8). Ces derniers ne confluent pas et à la métaphase I, comme à l'anaphase I, on se trouve en présence de deux fuseaux indépendants. Pendant ces périodes, on assiste encore à une légère augmentation du nombre de grains chromotropes rassemblés dans les asters et surtout à leur périphérie. Le reste du plasme périnucléaire n'en est pour autant pas dépourvu. On en trouve toujours au sein du vitellus; il s'agit ici essentiellement de formes 'jeunes'. Dès maintenant la morphologie des granules concentrés dans l'aire périnucléaire est donc nettement différente de celle des éléments épars dans le vitellus (cf. paragraphe 1).

A l'intercinèse des stades ultérieurs, la situation est celle que nous avons décrite sous le paragraphe 1. Au stade préprophasique la plupart des grains M mûrs vont se localiser aux deux pôles du noyau, zone dans laquelle les mitochondries vont également se concentrer, comme nous l'avons décrit antérieurement (Planche, fig. 10). A la métaphase (Planche, fig. 12) et à l'anaphase (Planche, fig. 11) on les trouve surtout à la périphérie des asters; quelques-uns s'observent cependant dans le reste du plasme périnucléaire.

Quant à la distribution des granules M au cours de la segmentation, nous n'avons pas remarqué qu'un des blastomères soit favorisé. D'un animal à l'autre, les œufs peuvent être plus ou moins riches en granules M. Ces variations sont-elles attribuables à des différences toujours inévitables dans la technique de fixation telles que la vitesse de pénétration du fixateur ou sont-elles réellement l'expression d'un état différent de la cellule? Nous espérons au cours de cultures d'œufs *in vitro* pouvoir éliminer le plus possible les variations dans les manipulations techniques.

A partir de la blastula âgée on a l'impression que le nombre de granules chromotropes diminue légèrement; ceci est particulièrement vrai pour ceux qui semblent néoformés au sein du vitellus. On en retrouve toujours dans l'aire périnucléaire, plus ou moins condensés aux pôles de la future mitose. C'est

ainsi qu'immédiatement avant la gastrulation, alors que les noyaux forment une couronne superficielle, la plupart des granules M mûrs sont situés de part et d'autre des noyaux, aux extrémités de l'axe dans lequel se situeront les fuseaux et qui est parallèle à la surface du germe. Rares sont les granules chromotropes jeunes distribués entre les grains du vitellus. Pendant la gastrulation enfin les éléments chromotropes se raréfient et finissent par disparaître complètement.

DISCUSSION

La coloration des œufs d'*Artemia salina* au bleu de toluidine a attiré l'attention sur la présence de granules M chromotropes situés tant dans le plasme périnucléaire qu'entre les grains de vitellus. Une fois repérés, ils ont pu être retrouvés par d'autres colorations et notamment par des techniques histochimiques. Les résultats de cette dernière investigation feront l'objet d'une partie suivante de ce travail.

Les éléments les plus typiques se trouvent dans le plasme périnucléaire et se concentrent à la périphérie des asters lors de la mitose. Ils se présentent comme une flammèche plus ou moins régulière à métachromasie γ, à laquelle est accolé au moins un grain bleu sombre. Mais entre le vitellus on trouve en outre des grains dont la taille est égale ou légèrement inférieure à celle d'une plaquette vitelline, et qui se colorent en bleu foncé, en bleu violacé ou en violet franc (métachromasie β) mais contiennent une ou plusieurs inclusions rouges (métachromasie γ). Toutes les transitions existent entre ces derniers grains et les éléments chromotropes du plasme périnucléaire. Cette évolution semble être accompagnée d'une migration vers le plasme périnucléaire, où l'on trouve les éléments les plus évolués.

A l'origine de cette évolution se trouve un grain de vitellus : les micro-vacuoles qu'on y observe, commencent par se remplir de la substance qui donne la métachromasie γ. Mais pour l'origine vitelline ne plaident pas seulement l'aspect et la localisation des plus jeunes éléments chromotropes. Lorsque l'on suit les œufs en maturation on est frappé de constater que jamais un oöcyte, qui n'a pas encore ébauché sa vitellogénèse, ne contient de granules chromotropes. Par contre, dès que la vitellogénèse s'installe on voit apparaître entre les grains de vitellus les premiers éléments chromotropes jeunes.

Il semble bien que les grains chromotropes aient quelque chose à voir avec l'activité mitotique au cours de la segmentation. Les éléments les plus évolués ne se condensent pas seulement dans le plasme périnucléaire, mais, lors des mitoses, plus en particulier à la périphérie des asters. Leur formation qui s'installe dès le début de la vitellogénèse, régresse sensiblement vers la fin de la segmentation. Leur origine à partir du vitellus pourrait peut-être faire supposer qu'ils apportent du matériel énergétique nécessaire aux mitoses successives.

Il est en outre assez curieux de constater que les mouvements des granules M du plasme périnucléaire se synchronisent avec ceux des mitochondries, que l'une

de nous a décrit antérieurement (N. Fautrez-Firlefyn, 1960). La disposition des mitochondries autour du noyau interphasique est identique à celle des granules M. Elles se groupent vers les pôles du noyau au moment du dédoublement du centre cellulaire, qui se retrouve toujours parmi elles, et s'éloignent ensuite dans des 'couloirs' bien délimités, du noyau prophasique pour se regrouper autour des centres. C'est en général dans ces aires mitochondriales que l'on retrouve le plus de granules M.

Si l'apparition des grains chromotropes semble se faire au moment où les mitoses vont se succéder normalement à un rythme rapide, il faut cependant observer que, si l'on arrête l'entrée en maturation (femelles vierges), la formation de grains chromotropes se poursuit de manière indépendante au sein du vitellus.

On peut enfin se demander si les granules chromotropes trouvés chez *Artemia salina* peuvent être homologués aux granules α et β décrits dans les œufs d'espèces variées par les embryologistes de l'école bruxelloise. Il est évident qu'à part leur caractère chromotrope, ils s'en rapprochent par une série de propriétés. Comme nous n'avons aucun renseignement sur leur comportement *in vivo* vis-à-vis du bleu de toluidine, il nous semble impossible de les homologuer sans plus. S'il est vrai que les grains jeunes trouvés parmi les plaquettes vitellines pourraient être rapprochés des grains α et les granules mûrs situés dans le plasme périnucléaire assimilés aux grains β, la situation chez *Artemia salina* serait, semble-t-il, assez exceptionnelle. Alors que les auteurs bruxellois insistent sur le fait que les granules α et β sont des entités nettement différentes, nous trouvons en effet chez *Artemia salina* tous les *stades de transition* à partir de l'élément chromotrope jeune, issu d'une plaquette vitelline, jusqu'au grain le plus évolué situé dans l'aster.

Il faut d'ailleurs faire observer que d'importantes variantes spécifiques ont été relevées quant au comportement des éléments métachromatiques. Une distinction radicale entre granules α et β a été trouvée par Pasteels (1955) et par Pasteels & Mulnard (1957). Mais chez *Chaetopterus*, Mulnard (1959) décrit une troisième structure, qui pourrait n'être que le matériel chromotrope passant des granules α aux granules β.

Chez les mammifères comme chez les Ascidies, Dalcq (1952, 1957) n'est pas parvenu à retrouver deux groupes nettement distincts de granules métachromatiques. Chez *Ascidiella aspersa*, cet auteur décrit, à l'approche de la gastrulation, des corpuscules qui résultent de la rupture des plaquettes vitellines et deviennent métachromatiques. Cette observation se rapproche des données que nous avons recueillies chez *Artemia*, où cependant tout le matériel métachromatique trouve son origine dans le vitellus, selon le processus que nous venons de décrire.

RÉSUMÉ

La coloration au bleu de toluidine sur matériel fixé permet de montrer chez *Artemia salina* la présence de granules chromotropes dans l'œuf en vitellogénèse, en maturation et en segmentation. Ces granules trouvent leur origine dans

certaines plaquettes vitellines. Jeunes, ils se trouvent entre le vitellus, mais tout en 'mûrissant' ils vont s'accumuler, à partir du moment de la copulation des pronucléi, dans le plasme périnucléaire. Pendant les mitoses, ils sont essentiellement localisés à la périphérie des asters. Leur nombre diminue vers la fin de la segmentation. Alors qu'ils semblent jouer un rôle dans les mitoses, leur formation progresse cependant de manière indépendante chez les femelles vierges où les œufs restent bloqués à la métaphase de la première division de maturation.

SUMMARY

Toluidine blue staining of fixed eggs of *Artemia salina* reveals during vitellogenesis, maturation, and segmentation the presence of metachromatic granules. These granules originate in certain yolk platelets. Scattered throughout the yolk, they show a series of progressive transformations while migrating into the perinuclear plasm, which they reach at the time of copulation of the pronuclei. During the cleavage divisions they are principally located around the asters. New elements continue to be formed within the yolk until the end of cleavage, when metachromatic elements disappear from the egg. Though they have obviously something to do with the rapid succession of mitoses during cleavage, their formation continues nevertheless in virgin females whose eggs are blocked in metaphase of the first maturation division.

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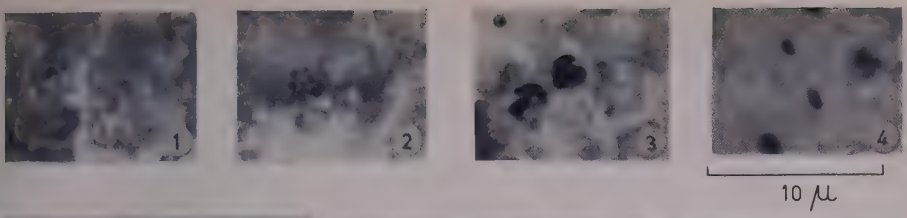
EXPLICATION DE LA PLANCHE

Fragments d'œuf d'*Artemia salina* fixés à l'AFA et colorés au bleu de toluidine. Par l'usage d'un filtre vert les grains rouges (métachromasie γ) apparaissent en noir. A noter que la fixation à l'AFA est peu favorable pour la mise en évidence des asters, qui se soupçonnent par leurs irradiations entre les grains de vitellus. La mise au point fut essentiellement faite sur les granules M.

Figs. 1–4. Formation des granules M à partir du vitellus (à fort grossissement).

Fig. 1. Micro-vacuoles refringeantes et 'vides' au sein de granules de vitellus.



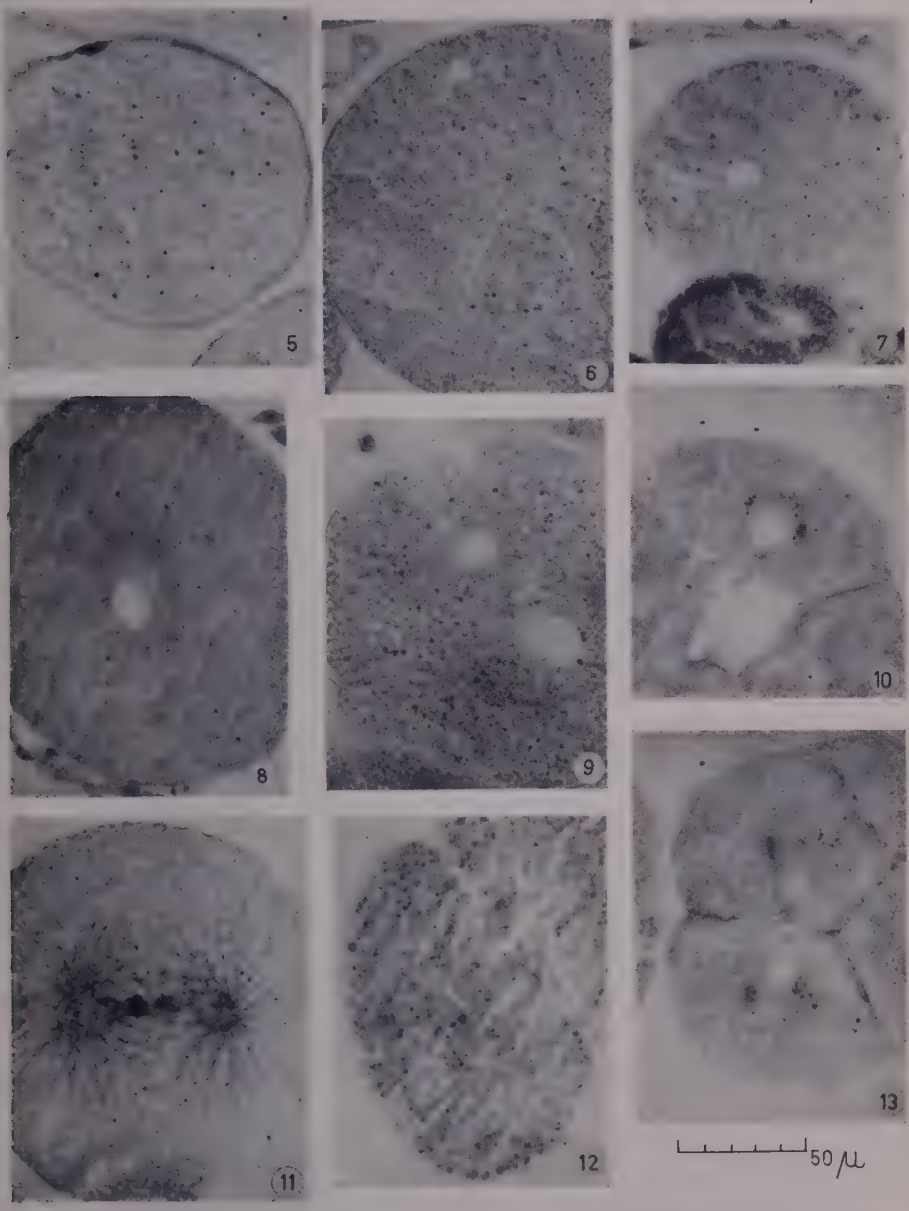


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FIG. 2. Les micro-vacuoles se remplissent de substance chromotrope.

FIG. 3. Les micro-vacuoles confluent au sein du grain de vitellus qui se rétracte.

FIG. 4. Grains M mûrs à la périphérie du plasme périnucléaire. Substance à métachromasie γ en noir; les granules bleuâtres, qui y sont accolés, apparaissent moins foncés.

FIG. 5–7. Stades de maturation et de fécondation, pendant lesquels les granules M 'jeunes' restent irrégulièrement distribués dans la masse vitelline.

FIG. 5. Métaphase de la 1^{e} division de maturation (image mitotique vers le haut de l'œuf).

FIG. 6. Le pronucléus se gonfle encore près de la surface ovulaire.

FIG. 7. Le pronucléus suivi d'un petit aster spermatique (vers le haut) s'enfonce dans l'œuf.

FIG. 8. Accolement des pronucléi au sein d'un large plasme périnucléaire à la périphérie duquel des grains M mûrs viennent s'accumuler.

FIG. 9. Stade IV, intercinèse. Granules M jeunes distribués entre le vitellus et granules M mûrs (plus petits) situés à la périphérie du plasme périnucléaire.

FIGS. 10–13. Situation des grains M au cours de la mitose.

FIG. 10. Stade VIII, début de la prophase. Les granules M se concentrent vers les 2 pôles.

FIG. 11. Stade V, anaphase. Les granules M se situent surtout dans les deux asters.

FIG. 12. Stade II, métaphase. Les granules M se trouvent également au niveau des deux asters (œuf cultivé *in vitro*).

FIG. 13. Stade VIII. Fin de la télophase. Les grains M semblent quitter la région périphérique des asters pour aller entourer les noyaux reformés.

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Sur l'origine du sang et des vaisseaux chez *Lebistes reticulatus* (Téléostéen)

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AVEC UNE PLANCHE

INTRODUCTION

DANS l'ensemble des Vertébrés, l'ébauche du sang et des vaisseaux est constituée par une portion du mésoblaste située en contact avec l'entoblaste et à distance des grands organes axiaux. Qu'il s'agisse de l'îlot sanguin médio-ventral des formes holoblastiques si typique chez les Amphibiens, ou des îlots de Pander–Wolff extra-embryonnaires des formes téloblastiques, le lieu d'origine du sang et des vaisseaux se localise toujours aussi loin que possible des réceptacles médio-dorsaux de substances morphogénétiquement actives, entre l'entoblaste et la splanchnopleure. Il est classiquement admis depuis les travaux de Swaen & Brachet (1899, 1901) que les Téléostéens forment une exception à cette règle générale. Dans leurs germes pourtant téloblastiques l'ébauche sanguine est en effet intra-embryonnaire et représentée par la 'masse intermédiaire', amas mésoblastique situé entre les somites et le bord médian des lames latérales.

Les observations que nous relatons ici montreront que cette règle n'est du moins pas aussi générale qu'on ne l'énonce habituellement.

MATÉRIEL ET TECHNIQUES

Nous nous sommes adressée à l'œuf de *Lebistes reticulatus*, Téléostéen appartenant à l'ordre des Cyprinodontiformes vivipares et à la famille des Poeciliidae.

L'obtention d'œufs arrivés aux stades désirés est assez laborieuse. Chez ces animaux vivipares, il est en effet impossible d'estimer l'âge des embryons à partir du moment du rapprochement des sexes. Les femelles, isolées des mâles après la ponte, redeviennent en effet gravides à deux ou trois reprises; les diverticules du canal ovarien fonctionnent comme autant de réceptacles séminaux (Stolk, 1950). Afin d'augmenter les chances d'obtenir des stades jeunes du développement embryonnaire nous avons sacrifié les poissons par décapitation entre 15 jours et 3 semaines après la dernière ponte.

Par une large laparatomie médiane l'ovaire est exposé et les œufs sont

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soigneusement libérés. Chacun d'eux est observé au microscope de Greenough. Cet examen *in vivo* permet de repérer aisément la présence de sang rouge, chargé d'hémoglobine, dans les vaisseaux extra-embryonnaires. Avant que la présence d'hémoglobine ne soit décelable à la loupe, on peut se rendre compte de l'existence d'une circulation en dirigeant un fin spot lumineux sur la région cardiaque; dès que les contractions cardiaques se sont installées, la lumière réfléchie par la surface brillante du germe est rythmiquement déviée.

Après cet examen, l'œuf est fixé au Zenker. Après quelques minutes l'embryon se distingue à travers la membrane folliculeuse par une coloration blanche, qui tranche fortement sur le vitellus; à ce moment il devient aisé d'estimer son âge en déterminant sa longueur, en comptant le nombre de ses somites et en évaluant la partie de l'œuf recouverte par le mouvement d'épibolie du blastodisque.

Remarquons qu'au moment du sacrifice de la femelle les divers œufs fécondés peuvent avoir atteint des stades de développement très différents.

Après une fixation de 6 à 12 heures les œufs sont lavés, déshydratés et inclus à la paraffine. Aucune tentative n'a été faite, ni avant ni après fixation, pour extraire le germe de son follicule, ni pour séparer l'embryon, et ceci afin d'épargner la partie extra-embryonnaire du blastodisque.

Soigneusement orientés, les germes furent débités en coupes transversales sériées d'une épaisseur de 7 μ. Les colorations qui nous ont donné le plus de satisfaction sont l'hémalun-érythrosine d'une part, le Kernechtrot–Vert lumière d'autre part. La réaction de Unna–Brachet a également fourni des indications précieuses. L'hémoglobine fut enfin mise en évidence par la réaction pseudo-peroxydasique à la benzidine (Lison, 1953). Parmi un matériel assez abondant nous n'avons retenu que les germes coupés en série d'une manière convenable. Nous les avons groupés plus ou moins arbitrairement en 6 stades.

Le stade I va de 0 à 2 somites. Nous disposons de 5 embryons à 0 somites dont deux sont colorés à l'hémalun-érythrosine, deux selon la technique de Unna–Brachet et un à la benzidine. Parmi les 3 embryons à 2 somites, deux sont colorés selon Unna–Brachet et un à l'hémalun-érythrosine.

Le stade II groupe les embryons qui comptent de 5 à 7 somites. Un embryon de 5 somites est coloré au Kernechtrot–Vert lumière, un second de 6 somites selon l'Unna–Brachet et des embryons de 7 somites un exemplaire fut coloré à l'hémalun-érythrosine et un à la benzidine.

Au stade III appartiennent les embryons de 8 à 10 somites. Un embryon de 8 somites est coloré à l'hémalun-érythrosine, un premier de 9 somites au Kernechtrot–Vert lumière et un second selon la technique de Unna–Brachet. Enfin nous disposons de deux exemplaires à 10 somites colorés respectivement à l'hémalun-érythrosine et au vert de méthyle-pyronine selon Unna–Brachet.

Le stade IV réunit les embryons de 12 à 14 somites. Un embryon à 12 somites et un à 13 somites sont colorés selon le procédé de Unna–Brachet, un deuxième à 12 somites ainsi qu'un exemplaire à 14 somites par la réaction pseudo-peroxydasique à la benzidine.

Le stade V va de 15 à 17 somites. Nous disposons d'un exemplaire à 15 somites coloré à l'hémalun-érythrosine, d'un embryon à 16 somites coloré au vert de méthyle-pyronine selon Unna–Brachet, et d'un embryon de 17 somites coloré à la benzidine.

Enfin le stade VI groupe les embryons de 18 à 20 somites. Nous disposons de 3 embryons à 18 somites dont l'un est coloré à l'hémalun-érythrosine, un second selon le procédé de Unna–Brachet et le troisième par la réaction pseudo-peroxydasique à la benzidine. Un embryon de 19 somites est coloré selon Unna–Brachet et un dernier de 20 somites à la benzidine.

OBSERVATIONS PERSONNELLES

Stade I: Il s'agit d'embryons de 0 à 2 somites mesurant 0·64 à 0·83 mm.; le bord d'enveloppement n'a pas encore atteint l'équateur de l'œuf.

Dans ces embryons on ne trouve à la place de la masse intermédiaire que quelques petites cellules d'aspect mésenchymateux; certaines d'entre elles atteignent la ligne médiane (Planche, fig. 1). A la partie ventrale du bord d'enveloppement du blastodisque on rencontre cependant sous le recouvrement épiblastique des amas de quelques cellules qui semblent s'en détacher. Les plus petites sont arrondies; leur noyau n'est entouré que d'une mince couche de cytoplasme. Les plus grandes sont arrondies ou polygonales. Leur cytoplasme est nettement basophile à la coloration de Unna–Brachet (Planche, fig. 2). Dans la moitié dorsale du bord d'enveloppement ces amas font défaut. Comme nous le montrera leur évolution ultérieure, il s'agit de véritables îlots sanguins.

Stade II: Embryons de 4 à 7 somites mesurant 0·89 à 1 mm. Le bord d'enveloppement reste toujours en deçà de l'équateur.

La situation dans le bord d'enveloppement a fortement changé. Les cellules arrondies qu'on voyait apparaître en petites masses isolées au stade précédent ont intensivement proliféré. Il s'est ainsi constitué des amas importants de jeunes érythroblastes, qui commencent à confluer pour constituer un anneau continu. Le nombre d'éléments en mitose est d'ailleurs très important. Le bord d'enveloppement est envahi dès maintenant sur tout son pourtour. La fig. 3 de la Planche montre sa partie postérieure de part et d'autre du nœud terminal. Dorsalement, l'ébauche sanguine s'enfonce de chaque côté de la ligne médiane dans la partie latérale profonde du nœud terminal. A ce niveau les érythroblastes sont moins visibles et moins différenciés que dans le bord d'enveloppement du blastodisque; ils sont coincés entre l'embryon et le vitellus. A l'extrémité antérieure du nœud terminal les deux coulées droite et gauche viennent se rejoindre sur la ligne médiane entre la chorde et l'entoblaste. Ici encore des cellules s'arrondissent et se différencient en érythroblastes (Planche, fig. 4). Cette ébauche impaire est vers l'avant en continuité avec les éléments issus de la masse intermédiaire. Ces derniers se trouvent dans la partie du tronc où les somites se sont déjà individualisés; ils ont atteint la ligne médiane tout en gardant leur aspect mésenchymateux. Dans la région du cœur la lame cardiaque

est étranglée par le mésoblaste ventral qui se dirige vers la ligne médiane; ainsi se forme la première ébauche de l'épi-myocarde. Dans la région somitique comme dans la région cardiaque, les cellules ne présentent aucune différenciation érythroblastique.

La réaction de l'hémoglobine est négative chez des embryons possédant 7 somites.

Stade III: Embryons de 8 à 10 somites mesurant 1·1 à 1·2 mm. Le bord d'enveloppement du blastodisque atteint l'équateur.

A ce stade la situation de l'ébauche sanguine n'a guère changé par rapport au stade précédent. Les éléments sanguins, fortement basophiles au Unna-Brachet, prolifèrent et s'accumulent le long du bord d'enveloppement du blastodisque et atteignent de chaque côté le nœud terminal. Le mésoblaste n'est guère différencié à ce niveau. Il semble bien que c'est entre les futures lames latérales et la masse vitelline que l'ébauche sanguine s'enfonce sous l'embryon. A ce niveau elle est représentée par des érythroblastes très basophiles isolés ou groupés par amas de 3 ou 4 cellules (Planche, fig. 5). A la partie antérieure du nœud terminal les ébauches paires confluent sur la ligne médiane entre la chorde et l'entoblaste (Planche, fig. 6); elles y forment une masse continue de futurs globules rouges qui, au niveau des derniers somites individualisés, se continue vers l'avant par la lame vasculaire issue des masses latérales. Cette dernière reste formée de cellules d'aspect mésenchymateux, petites et peu basophiles. On n'y voit aucune différenciation dans la direction sanguine mais peut-être déjà un certain arrangement en parois vasculaires.

Stade IV: Embryons de 12 à 14 somites, mesurant 1·3 à 1·4 mm. Le bord du blastodisque dépasse l'équateur de l'œuf.

A ce stade l'ébauche extra-embryonnaire se compose de vaisseaux délimités par des cellules endothéliales. La plupart de ces cavités sont bourrées d'érythrocytes. On retrouve cependant des cellules sanguines nettement différenciées en dehors des vaisseaux. La taille et la basophilie cytoplasmique de ces cellules sanguines sont très variables. Elles sont fréquemment en mitose.

Remarquons qu'à partir de ce stade les deux branches de l'ébauche qui vont glisser sous le nœud terminal, sont reliées par une large branche anastomotique, qui passe en arrière de celui-ci.

Dans l'embryon même un vaisseau axial s'est constitué: sa lumière est aplatie en avant (Planche, fig. 7) tandis qu'elle devient béante et remplie d'érythrocytes vers l'arrière (Planche, figs. 8 et 9). L'extrémité postérieure, en continuité avec les vaisseaux qui glissent sous le nœud terminal, est remplie d'érythrocytes.

A ce stade la réaction de l'hémoglobine est positive dans les érythrocytes extra-embryonnaires alors qu'elle reste négative dans les éléments sanguins situés dans le vaisseau axial de l'embryon.

Stade V: Embryons de 15 à 17 somites, mesurant de 1·4 à 1·5 mm. Les $\frac{3}{4}$ du globe vitellin sont recouverts par le blastodisque.

La masse sanguine extra-embryonnaire s'est fortement élargie surtout au

niveau d'un épais croissant situé près de la partie ventrale du bord d'enveloppement. Elle est devenue tellement large, qu'elle s'étend jusque près de la tête et se prolonge latéralement autour de la vaste cavité péricardique. La plupart des érythrocytes sont situés dans des vaisseaux délimités par un mince endothélium: l'ensemble repose directement sur le vitellus et est recouvert par l'épiblaste. Sur la ligne médiane cet amas extra-embryonnaire se met en continuité avec le cœur. Ce dernier n'a pas encore effectué sa rotation et se présente comme un vaisseau médian situé sous la tête et sous le cou. Il contient dès maintenant des globules rouges et est entièrement entouré d'épi-myocarde.

Plus en arrière l'aorte s'est déployée dans sa partie antérieure, qui contient quelques rares érythrocytes. Dans sa partie caudale elle est moins bourrée de cellules sanguines qu'au stade précédent. Les vaisseaux bifurqués sous la partie caudale du tronc sont remplis de sang comme la partie de l'anneau extra-embryonnaire qu'ils continuent. Remarquons cependant que la partie dorsale de cet anneau ne contient plus que de rares érythrocytes, sauf tout près du nœud terminal (Planche, fig. 10).

En dehors des grands vaisseaux axiaux, on voit se former dans la tête des cavités entourées de cellules endothéliales et contenant du plasma et des cellules sanguines; il s'agit de vaisseaux souvent encore discontinus.

Comme dans les stades antérieurs nous n'avons vu se former que des vaisseaux dans la tête et la partie antérieure du tronc, il nous semble vraisemblable que les cellules sanguines y sont amenées de la zone extra-embryonnaire. Ceci expliquerait d'ailleurs pourquoi la partie postérieure de l'anneau extra-embryonnaire se vide grandement de son sang. Malgré le fait que nous n'avons jamais vu battre le cœur avant le stade de 17 somites, il n'est pas impossible que déjà maintenant une certaine circulation se soit installée.

La réaction de l'hémoglobine est positive dans les cellules situées dans les vaisseaux intra- et extra-embryonnaires.

Stade VI: Les embryons de 18 à 20 somites mesurent de 1·5 à 1·7 mm. L'épibolie du blastodisque est pratiquement achevée: l'examen à la loupe révèle la présence de sang rouge, chargé d'hémoglobine dans les larges vaisseaux qui s'étalent sur la masse du vitellus. Des pulsations rythmiques du cœur s'observent aisément.

La situation du système circulatoire n'a guère changé dans sa constitution générale par rapport au stade précédent; toutefois le nombre de globules rouges est bien plus élevé, les parois endothéliales sont mieux constituées. Des cellules mésenchymateuses s'ordonnent pour délimiter de nouvelles cavités. Autour de la cavité péricardique, deux larges vaisseaux se sont formés; ils suivent le bord postérieur du péricarde pariétal pour s'engager ensuite sous le mésoblaste ventral tout juste en avant des premiers somites individualisés.

Dans les érythrocytes le cytoplasme a augmenté de volume. Il reste basophile.

DISCUSSION

Nombreuses sont les espèces de Téléostéens chez lesquelles le développement

embryonnaire de l'appareil circulatoire et du sang fut étudié. Nous ne citons ici que quelques travaux devenus classiques. Parmi les formes les plus primitives, les Clupéiformes, *Salmo* et *Esox* furent examinés par Ziegler (1887) tandis que Swaen & Brachet (1899, 1901) ont étudié *Clupea* et la Truite. Parmi les Cypriniformes ces derniers auteurs se sont adressés à *Leuciscus*. Des Béloniformes furent examinés par Wenchebach (1886), par Swaen & Brachet (1901). Entre les Perciformes, qui représentent le type Acanthoptérygien dans toute sa plénitude, *Perca* fut étudiée par Wenchebach (1886), *Caranx*, *Callionymus* et *Trachinus* le furent par Swaen & Brachet (1901). Dans l'ordre des Pleuronectiformes, poissons plats encore plus évolués, Swaen & Brachet (1901) ont décrit l'évolution des masses intermédiaires chez *Solea* et *Pleuronectes*.

Dans toutes les formes chez lesquelles ces auteurs ont pu suivre l'érythropoièse avant l'éclosion, ils virent sang et vaisseaux provenir de la masse intermédiaire d'Oellacher, située entre le scléromyotome et les lames latérales. Cette masse s'isole et glisse sous le scléromyotome pour gagner la ligne médiane entre la chorde et l'entoblaste.

Son degré de développement semble très différent selon les espèces. D'après Wenchebach (1886) les éléments qui la constituent se sont déjà rassemblés sur la ligne médiane au moment où l'on commence à les distinguer chez les Béloniformes.

Si tous les auteurs cités sont d'accord à admettre que la masse intermédiaire fournit le sang, leurs avis sur l'origine des vaisseaux sont plus divergeants. S'ils font provenir la veine médiane de la masse intermédiaire, qui pour Ziegler (1887) ne serait qu'un vaisseau bourré d'érythrocytes, ils attribuent souvent à l'aorte une origine mésenchymateuse. En ce qui concerne l'origine des vaisseaux il n'existe d'ailleurs pour Ziegler (1887) aucune différence fondamentale entre la masse intermédiaire et le tissu conjonctif, le 'Bildungsgewebe'.

Depuis les travaux de Swaen & Brachet (1899, 1901) on considère cependant classiquement les masses intermédiaires comme les ébauches communes à l'aorte, la veine axiale et les érythrocytes. Elles formeraient en outre par l'intermédiaire de cellules migratrices, qui s'en détachent, le reste du système vasculaire intra- et extra-embryonnaire. Ces auteurs reviennent en quelque sorte à la conception de His (1876) selon laquelle il existe dès le début une distinction nette entre le tissu conjonctif et l'ébauche vasculo-sanguine (angioblaste).

Chez les espèces à œufs pélagiques Swaen & Brachet (1901) ont observé que les masses intermédiaires ne fournissent que les vaisseaux. Les embryons ne possèdent pas de cellules sanguines au moment de l'éclosion. Le sang qui circule est au début totalement dépourvu d'érythrocytes. On ignore la provenance des globules rouges qui rempliront ultérieurement les vaisseaux.

La situation que nous avons trouvée chez *Lebistes reticulatus* est très différente de ce qui fut décrit chez les autres espèces de Téléostéens. Elle se rapproche beaucoup plus de ce que l'on observe dans les autres classes de Vertébrés à œufs télolécithiques. Une ébauche commune au sang et aux vaisseaux se localise en

dehors de l'embryon, le long du bord d'enveloppement du blastodisque. C'est dans la partie ventrale qu'elle se distingue et se différencie le plus précocement et qu'elle reste d'ailleurs le plus développée. Sous le nœud terminal elle se continue dans l'homologue de la masse intermédiaire, qui se différencie en une lame vasculaire dans le tronc et en une lame cardiaque dans le cou.

Ces lames sont très peu développées chez *Lebistes*; très précocement rassemblées sur la ligne médiane, elles ne sont formées que d'une traînée de cellules très mince et discontinue.

Quoique l'ensemble de cette ébauche donne origine tant aux vaisseaux qu'au sang, un double gradient semble s'y dessiner. Les éléments sanguins se constituent avant tout dans l'anneau péridiscal; leur différenciation se produit le plus précocement dans sa partie ventrale. Dans la partie invaginée avec le mésoblaste embryonnaire, on ne voit pratiquement plus se différencier de cellules sanguines. Pour les vaisseaux le gradient évolue en sens opposé. La masse intermédiaire ne forme pratiquement que des vaisseaux; dans la partie ventrale de l'anneau discal, les cellules endothéliales n'apparaissent que tardivement pour entourer les érythrocytes.

On pourrait comprendre par le fait que le blastopore est péridiscal chez les Téléostéens, qu'une partie dorsale de l'ébauche sanguine est invaginée avec le mésoblaste embryonnaire, contrairement à ce qui se passe dans les autres classes de Vertébrés à œufs télolécithiques et à blastopore intradiscal. Cette situation, moins exceptionnelle que ce qui fut décrit chez d'autres Téléostéens, rend une prospection plus large dans l'ensemble de la Classe indispensable. Mais quel que puisse en être le résultat, il nous semble que dès maintenant la situation trouvée chez *Lebistes reticulatus* permet de rattacher assez aisément l'origine 'classique' du sang chez les Téléostéens à ce qui est connu par l'ensemble des Vertébrés. On pourrait concevoir que primitivement l'ébauche du sang et des vaisseaux est extra-embryonnaire: par le fait que le blastopore est péridiscal, une partie en est invaginée avec le mésoblaste embryonnaire (situation *Lebistes*). Il suffirait d'imaginer une mutation, par laquelle seule la partie la plus dorsale (celle qui est invaginée) de l'ébauche vasculo-sanguine se développe pour arriver à la situation 'classique' chez les Téléostéens.

RÉSUMÉ

Chez le Téléostéen *Lebistes reticulatus* la première ébauche des érythroblastes est visible au stade de 2 somites. La zone d'érythropoièse se trouve dans la partie ventrale du bord d'enveloppement du blastoderme. Elle se répand rapidement le long de ce bord jusqu'à l'extrémité caudale de l'embryon où elle se continue dans les éléments de la masse intermédiaire de Oellacher.

A l'intérieur de l'embryon le sang ne se différencie qu'à partir du stade de 5 somites. Cette masse sanguine prendra la forme d'une Y renversée située dans la partie caudale du tronc. Les deux branches de cette Y se continuent de part et d'autre du bouton terminal dans la masse sanguine extra-embryonnaire qui

suit le bord d'enveloppement. Elles se dirigent vers la ligne médiane entre le vitellus et les lames latérales.

A partir du stade de 15 à 17 somites la circulation met toute la masse sanguine en branle. Les érythrocytes se déplacent à la surface du vitellus dans la direction de la portion veineuse du cœur. La fermeture du blastopore ne se produit que vers le stade de 18 somites, lorsque la circulation est déjà établie.

Ces observations ne correspondent pas avec celles qui ont été faites chez d'autres Téléostéens où la localisation de l'ébauche sanguine est uniquement située dans la partie dorsale du corps. Elles pourraient permettre de rattacher cette origine exceptionnelle des éléments vasculo-sanguins à la situation que l'on trouve dans toute la lignée des Vertébrés.

SUMMARY

The erythroblasts of the Teleost *Lebistes reticulatus*, when visualized with the Unna-Brachet technique, appear at the 2-somite stage of development. The earliest visible erythropoiesis is then located in the ventral part of the border of the blastodisc. Along this border it rapidly extends towards the posterior extremity of the embryo, where it appears continuous with the elements of the 'Intermediäre Zellenmasse' of Oellacher.

The blood-forming rudiment in the embryo itself becomes visible only at the stage of 5 somites, and takes the shape of an inverted Y whose long limb is localized in the midline of the posterior somitic region of the embryo; the two branches of the Y taking their origin in the posterior part of the extra-embryonic tissue on both sides of the terminal node.

Blood-circulation becomes apparent at the 15–17-somite stage, and at the surface of the yolk the blood-cells then move towards the venous side of the heart. The closure of the blastopore occurs around the stage of 18 somites, when the blood-circulation is already established.

These observations do not correspond with what is found in other Teleosts where the blood-forming rudiment appears to be situated only in the dorsal part of the body. It would now seem possible to relate this extraordinary origin to the situation which prevails in other Vertebrates.

REMERCIEMENT

Nous tenons à exprimer à M. le Professeur J. Fautrez nos sentiments de profonde gratitude. Nous lui devons non seulement le sujet de nos recherches mais c'est aussi grâce à ses conseils et à son aide que nous avons pu réaliser ce travail.

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EXPLICATION DE LA PLANCHE

FIG. 1. *Embryon de 2 somites.* Coupe transversale au niveau de la région somitique (Col.: hémalun-érythrosine). La masse intermédiaire est réduite à une traînée de cellules située entre le somite et les lames latérales et qui se continue déjà sous le somite vers la région de la chorde. Elle est nettement visible sur le côté gauche de l'embryon.

FIG. 2. *Fragment de la partie ventrale du bord d'enveloppement au stade de 2 somites* (Col.: Unna–Brachet). Quelques érythroblastes disposés en petits îlots.

FIGS. 3, 4. *Embryon de 5 somites.* Deux coupes transversales. (Col.: Kernechtrot–Vert lumière).

FIG. 3. Coupe transversale au niveau du bouton terminal: de part et d'autre de celui-ci la section intéresse les traînées vasculo-sanguines (plus fortement colorées) qui continuent l'ébauche située dans le bord d'enveloppement pour rejoindre plus en avant le matériel des masses intermédiaires.

FIG. 4. Coupe transversale au niveau de la partie somitique postérieure du même embryon. Les deux masses intermédiaires (plus développées qu'au stade à 2 somites) partent de la fente située entre le somite et le mésoblaste ventral et se rejoignent sur la ligne médiane en dessous de la chorde.

FIGS. 5, 6. *Embryon de 9 somites.* Deux coupes transversales (Col.: Unna–Brachet).

FIG. 5. Coupe transversale au niveau de la partie du tronc située immédiatement en avant du bouton terminal. Les masses intermédiaires sont encore latérales à ce niveau. Elles se distinguent des somites et des lames latérales par une basophilie légèrement plus intense (surtout nette à gauche).

FIG. 6. Coupe transversale du même embryon, située légèrement en avant de la précédente (dans la partie postérieure du tronc). Les deux masses intermédiaires forment des traînées de cellules aplaties qui se rejoignent à ce niveau sous la chorde.

FIGS. 7, 8, 9. *Embryon de 12 somites.* Trois coupes transversales d'un même embryon sériées d'avant en arrière (Col.: Unna–Brachet).

FIG. 7. Coupe transversale au niveau de la région somitique antérieure. Les éléments issus de la masse intermédiaire forment des amas de cellules d'aspect mésenchymateux et peu basophiles situés sous la chorde. (Elles ne forment à ce niveau que l'endothélium des vaisseaux).

FIG. 8. Coupe transversale au niveau de la région somitique moyenne. On distingue sur la ligne médiane la première ébauche de l'aorte, contenant des cellules sanguines. Les cellules issues de la masse intermédiaire et situées de part et d'autre de l'aorte seront à l'origine des veines cardinales.

FIG. 9. Coupe transversale au niveau de la région somitique postérieure. Volumineux amas vasculo-sanguin situé en dessous de la chorde et dans lequel on devine difficilement les limites de l'aorte et de la masse veineuse bourrées de cellules sanguines.

FIG. 10. *Coupe d'un vaisseau extra-embryonnaire au stade de 15 somites.* (Col.: Unna–Brachet.) La plupart des cellules sanguines présentent encore une forte basophilie.

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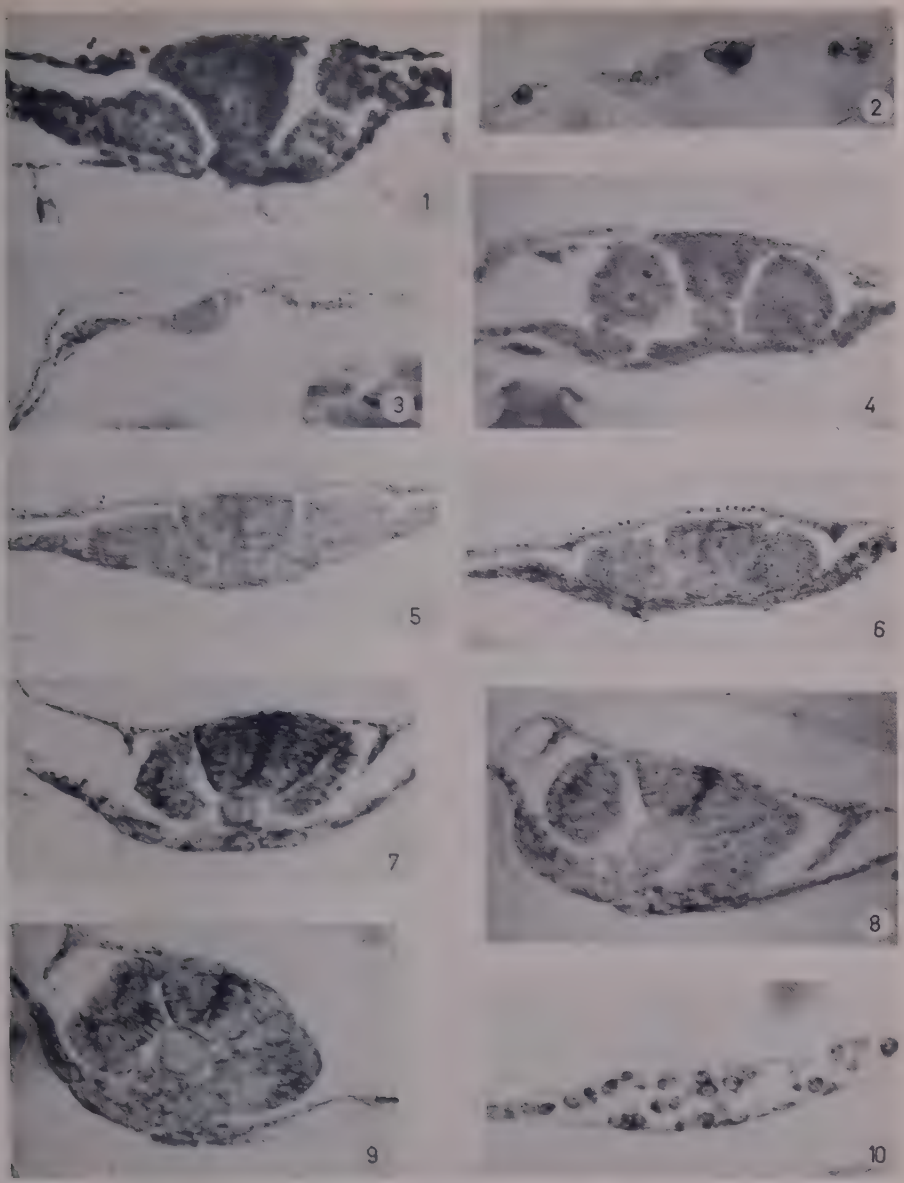

A. COLLE-VANDEVELDE

La Régénération caudale des Urodèles (Induction et réactivité du territoire)

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AVEC TROIS PLANCHES

INTRODUCTION

CHEZ les Batraciens, le processus de restitution le plus complet apparaît lors de la régénération de la queue, au cours de laquelle l'ensemble du système axial se reconstitue (Stefanelli, 1959). Pourtant, depuis longtemps, seule la régénération caudale des Anoures a été bien étudiée. Celle des Urodèles n'a été l'objet d'une analyse approfondie qu'à une époque récente (Holtzer, 1959). Les processus réparateurs dans les deux groupes d'Amphibiens sont loin d'être identiques. Il y a, en premier lieu, une différence de destinée entre la queue du tétard, organe larvaire disparaissant à la métamorphose, et l'appendice caudal d'une larve de salamandre ou de triton qui, après des remaniements mineurs, s'intègre dans le corps de l'adulte.

La régénération caudale des Anoures est caractérisée par la prépondérance de la croissance chordale qui allonge le régénérat (Needham, 1952). Seules se reforment les parties présentes au niveau d'amputation (Lüscher, 1946).

La situation est moins claire chez les Urodèles où la chorde dorsale, même embryonnaire, ne régénère jamais (Barfurth, 1891; Hadorn, 1952). Elle est remplacée, dans le régénérat, par un axe purement cartilagineux qui se métamérise en formant les vertèbres.

Le système nerveux joue un rôle important dans la régénération des vertèbres. Son action, partiellement élucidée en ce qui concerne des organes tels que la patte (Singer, 1954) ou le museau (Vallette, 1929), reste encore très obscure dans le cas de la queue. La présence simultanée de moelle épinière, de ganglions rachidiens et de nerfs, rend la désinnervation complète de la queue malaisée, sinon impossible.

Enfin, l'existence, au sein du territoire caudal, de parties d'origine et de structure diverses (Bijtel, 1958) pose le problème de leur participation respective à l'édification du régénérat.

Nous avons entrepris l'étude de quelques questions concernant la régénération de la queue chez les Urodèles:

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(*a*) La régénération caudale est-elle possible en absence de moelle épinière?

(*b*) Quel est le rôle joué par la moelle dans les processus régénérateurs de la queue? Peut-on la remplacer dans son action par d'autres éléments du système nerveux?

(*c*) Quelle est la réponse des différentes régions de la queue à l'induction? Le territoire 'queue' est-il, sur toute son étendue, morphogénétiquement isodyname?

MATÉRIEL ET TECHNIQUE

Nous avons utilisé au total cent-soixante-neuf larves de *Salamandra salamandra* Laur. et dix-huit larves de *Triturus cristatus* Laur. Ces dernières ont été capturées dans l'étang artificiel de l'Institut de Zoologie. On ignore leur ascendance et leur âge exact. Les Salamandres, elles, sont nées au laboratoire de femelles portantes provenant du Tessin (Suisse). Chaque femelle peut mettre bas une quarantaine de larves à la fois. Les larves de chaque portée sont élevées ensemble, dans des aquariums en verre dont l'eau est constamment oxygénée par une pompe à aire comprimé. Régulièrement nourries de *Tubifex* vivants, ces larves atteignent, en une vingtaine de jours, une longueur de corps (longueur totale, moins la queue) de 21 à 25 mm. Elles sont opérées à ce moment-là.

Technique des opérations. La narcose, profonde et prolongée, est obtenue par un séjour de dix minutes dans une solution de 1 : 5.000 de métacaïne (MS. 222). On opère les animaux, aseptiquement, dans la solution de Holtfreter, additionnée de 50.000 U.I. de pénicilline et de 50 mg. de streptomycine par litre.

La description détaillée des interventions est donnée, au début de chaque paragraphe, dans la partie expérimentale de cette étude. Après l'opération, on maintient les larves sous narcose légère et à une température d'environ +7 degrés, pendant douze heures. Ces précautions postopératoires permettent la consolidation des greffes et réduisent la mortalité.

Les larves opérées sont ensuite transférées dans des cristallisoirs individuels, remplis d'eau ordinaire aérée, gardées à la température de la chambre et nourries de *Tubifex*.

Les moignons, les régénérats et les greffes sont dessinés, à intervalles réguliers, à la chambre claire et photographiés avant la fixation. Ils sont fixés pendant douze heures au Bouin–Allen et débités en coupes sériées de 10 μ en vue de l'examen histologique. Les colorations suivantes ont été utilisées: Mallory–Azan, trichrome de Gabe et, pour mettre en évidence les fibres nerveuses, l'imprégnation argentique de Bodian.

EXPÉRIENCES

I. *Spécificité d'action de la moelle épinière*

1. *Greffes de queue sur le dos*

Il nous a semblé que la méthode la plus simple, pour étudier les potentialités morphogènes de la queue et les facteurs qui influent sur sa régénération, était la transplantation de cet organe à un endroit neutre.

Après l'amputation de l'extrémité caudale, nous prélevons dans la queue deux tranches, d'une épaisseur de trois millimètres, et nous les greffons sur le dos du même animal (fig. 1*a*). L'une des autogreffes est despinalisée. On enlève la portion de la moelle épinière qu'elle contient, en passant une aiguille de verre à travers son canal rachidien. La moelle est conservée dans l'autre tranche, qui sert ainsi de témoin.

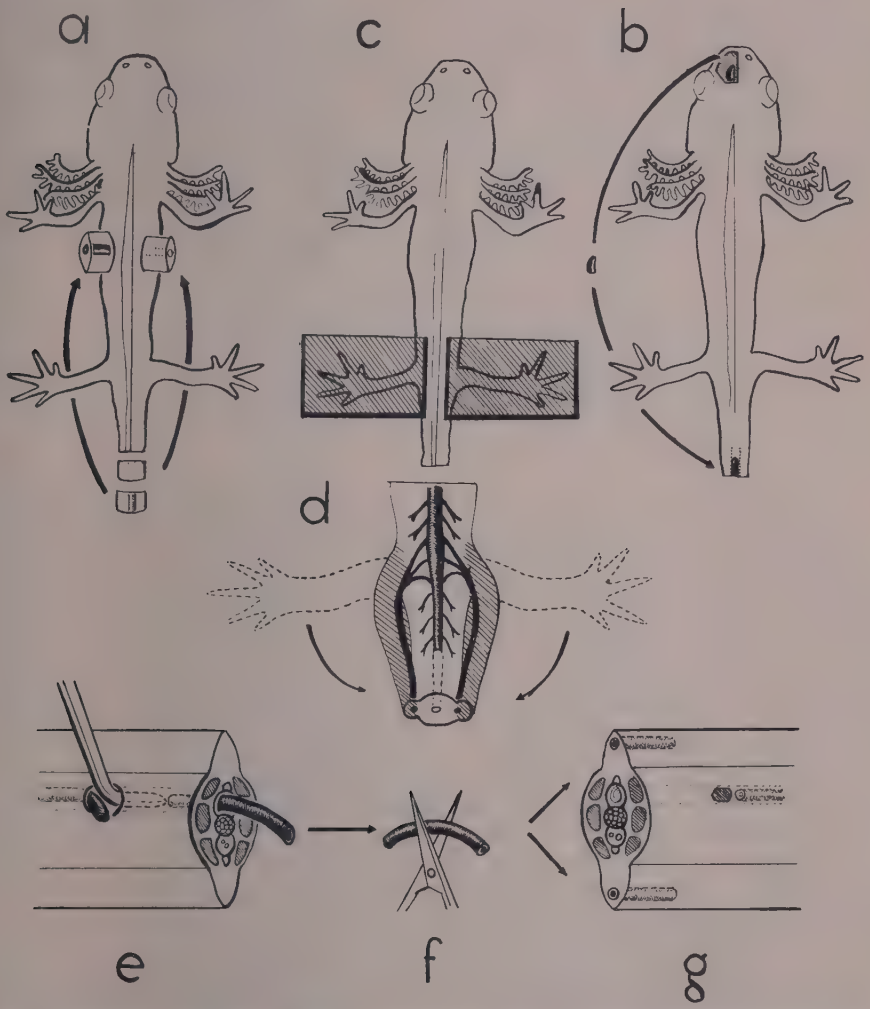

FIG. 1. Schéma des opérations: *a*, greffe de tranches de queue normales ou despinalisées sur le dos. *b*, despinalisation et greffe du télencéphale dans le canal rachidien évidé. *c*, irradiation localisée (hâchure) des pattes postérieures. *d*, incorporation des pattes irradiées (hâchure) dans le moignon caudal despinalisé, non irradié. En noir, la moelle et les nerfs spinaux. *e*, technique de la despinalisation et de la déviation latérale de la moelle restante. *f*, le fragment de moelle extirpé est divisé en deux. *g*, chacune des parties de la moelle est greffée dans la nageoire caudale (flèches) de la queue despinalisée.

Chaque greffe est simplement insérée, sans points de suture, dans une fente pratiquée sur le dos, de part et d'autre de la ligne médiane, un peu en arrière des membres antérieurs. Les surfaces d'amputation des deux tranches de queue sont identiques, seule la moelle épinière, présente dans l'une, absente dans l'autre, fait exception.

Nous avons pratiqué cette transplantation, unilatérale ou bilatérale, sur quarante-neuf animaux. Au total, trente-six greffes ont été retenues assez longtemps pour être prises en considération: quatorze témoins avec moelle épinière et vingt-deux despinalisées. Elles peuvent être classées, selon leur évolution, en trois catégories: (1) croissance et régénération des transplants; (2) greffes stationnaires; (3) régression des greffons, pouvant aller jusqu'à disparition complète. Les pourcentages sont indiqués dans le tableau 1.

TABLEAU 1

Greffes	*Évolution des greffons*			*Total*
	Croissance	*Stationnaires*	*Régression*	
Avec moelle .	7 (50%)	1 (7%)	6 (43%)	14
Sans moelle .	3 (14%)	1 (4%)	18 (82%)	22
TOTAL .	10	2	24	36

De toute évidence, une tranche de queue greffée sur le dos, qu'elle soit despinalisée ou non, peut régénérer, rester stationnaire ou s'atrophier. Néanmoins, il y a une nette différence dans les pourcentages de croissance ou de régression, entre les transplants qui ont une moelle et ceux qui en sont dépourvus; les premiers sont favorisés. La fig. 2 illustre ce fait. Au début, la greffe despinalisée, celle de droite, était plus volumineuse que la greffe témoin. Vingt jours plus tard, elle a notablement régressé, tandis que l'autre, tout en conservant sa taille, a régénéré une petite queue. La moelle épinière, indépendamment de son rôle morphogène, exerce sur les tissus transplantés une action conservatrice, trophique. Un fait analogue s'observe lors de la régénération de la patte pendant la période larvaire. Le nerf induit la formation d'un blastème et arrête ainsi, indirectement, la dédifférenciation et la régression du moignon (Schotté & Butler, 1941). Il est possible que la moelle épinière et, dans une moindre mesure, les constituants nerveux agissent de la même façon sur la queue.

L'observation par transparence et l'examen histologique des dix greffes ayant présenté une croissance régénérative révèlent les particularités suivantes dans la structure des régénérats:

Les transplants avec moelle donnent naissance à des régénérats de queue typiques, possédant deux nageoires et un axe squelettique et musculaire (Planche 1, fig. 6). Toutefois ces formations n'atteignent jamais la taille du régénérat caudal *in situ*.

Les greffons despinalisés forment des excroissances moins étendues, sans muscles ni squelette, constituées uniquement de tissu analogue à celui des nageoires (Planche 1, fig. 5).

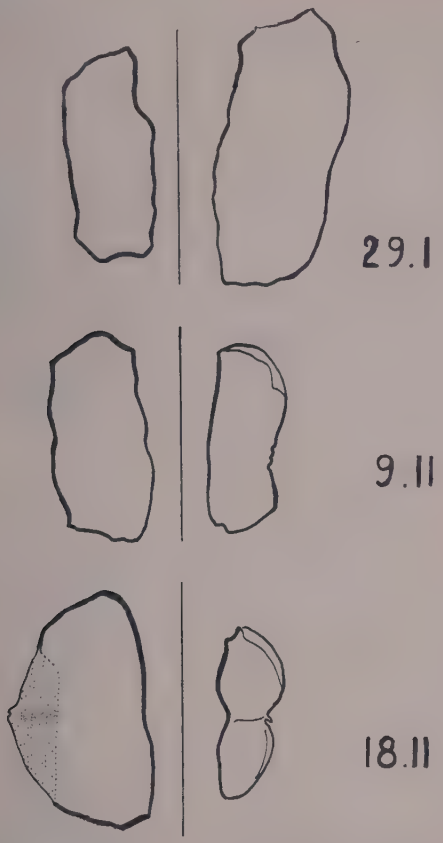

FIG. 2. Évolution de deux tranches de queue, greffées sur le dos. A gauche: tranche normale; à droite: tranche despinalisée. En pointillé: le régénérat. Les dates sont indiquées par les chiffres (jour, mois). Dessins à la chambre claire × 5,5.

Conclusion. Une tranche de queue greffée sur le dos régénère des structures caudales. Ce sont, soit de petites queues complètes, soit, en absence de moelle épinière, des organes caudiformes sans axe mésodermique.

En plus de son action morphogène, la moelle favorise le maintien et la croissance des greffons. Mais les régénérats, quels qu'ils soient, restent petits et les greffes peuvent présenter un état stationnaire et même une involution. Les conditions optimales nécessaires au développement du greffon, sont donc loin d'être réalisées.

2. *Despinalisation de la queue* in situ

Les aléas que présente la transplantation nous ont amené à pratiquer la suppression de la moelle épinière dans la queue *in situ*. La technique de l'opération laisse intacts la structure et les rapports fonctionnels du moignon caudal.

La queue est amputée transversalement à une distance de 10 mm. du cloaque. On pratique ensuite, à 3 mm. de la plaie, une petite ouverture latérale au niveau de la colonne vertébrale et, avec un scalpel très fin, on sectionne la moelle épinière. En faisant passer, à travers cet orifice, dans le canal rachidien, une aiguille de verre convenablement calibrée et courbée, on pousse vers l'extérieur le segment isolé de la moelle (fig. 1*e*). On introduit alors dans la fente latérale un fragment de peau, sorte de bouchon qui empêchera la régénération de la moelle à l'intérieur du canal rachidien évidé. Le moignon caudal ainsi préparé possède intacts tous les éléments de la queue, mais il est dépourvu de moelle épinière sur une longueur d'environ quatre vertèbres.

Sur soixante-quinze larves opérées et ayant survécu, neuf seulement ont présenté une régénération de la moelle à travers le moignon despinalisé. Les despinalisations permanentes, vérifiées histologiquement, sont au nombre de soixante-six (88 pour cent).

La régénération d'une queue typique dans ces soixante-six cas a toujours fait défaut. Seul un organe caudiforme a été formé. Tandis qu'extérieurement il simule un régénérat caudal de petite taille (Planche 1, fig. 7, 8), sa structure interne est semblable à celle d'une nageoire. Il est totalement dépourvu de musculature métamérique et de cartilages vertébraux (Planche 3, fig. 25). Par contre, il possède une riche vascularisation et des fibres nerveuses y pénètrent et le parcourent (Planche 3, fig. 22).

Dans une note précédente (Kiortsis, Uehlinger, & Droin, 1959), nous avons étudié la croissance de ces organes caudiformes, en la comparant à celle des régénérats normaux. Il ressort de cette étude que les régénérats hypotypiques des queues despinalisées présentent des courbes de croissance, en longueur et en volume, en tous points analogues à celles des normaux. Seul le taux de croissance de ces derniers est deux fois plus grand.

Nous nous sommes demandé si ces formations qui sont, malgré tout, de véritables régénérats représentaient des queues hypotypiques, dont la différenciation interne a avorté par manque d'innervation adéquate, ou si elles résultaient de la croissance simultanée et de la fusion des deux nageoires.

En observant les premiers stades de la régénération après amputation, on est enclin à admettre la première de ces hypothèses. En effet, le blastème régénérateur apparaît sur toute la surface de la plaie et rien ne distingue un très jeune régénérat de queue despinalisée d'un autre, formé sur une queue normale. Mais, pour des raisons que nous exposerons plus loin, cette hypothèse doit être rejetée. Ce sont les tissus des nageoires, et eux seuls, qui sont à l'origine de ces organes caudiformes, nés sur une queue despinalisée.

3. *Régénération caudale en présence des ganglions rachidiens*

Après despinalisation du moignon caudal, par évidement du canal rachidien, les nerfs, coupés de leurs connexions centrales, subissent la dégénérescence wallérienne. Seuls les ganglions rachidiens, isolés mais contenant des neurones, survivent. Nous avons constaté leur présence, au voisinage immédiat du niveau d'amputation, dans plus d'un cas. Deux à trois mois après la despinalisation, l'apparence de ces ganglions est restée saine. Ils émettent parfois des faisceaux de fibres qui se dirigent vers le régénérat (Planche 2, fig. 12). Mais, malgré cette innervation, le régénérat est un organe caudiforme, sans axe squelettique et sans musculature. Les ganglions rachidiens et leurs fibres n'ont pu remplacer la moelle épinière dans son action inductrice. C'est le contraire de ce qui se passe dans la patte désinnervée, dont Kamrin & Singer (1959) ont restitué les potentialités régénératives par implantation d'un ganglion spinal.

Nos observations ruinent complètement l'ancienne hypothèse de Locatelli (1929) selon laquelle la régénération de la queue se ferait par le truchement des ganglions rachidiens, eux-mêmes régénérés à partir de la moelle.

4. *Transplantation de cerveau dans une queue despinalisée*

La seule réserve à faire aux conclusions du paragraphe précédent serait que les ganglions rachidiens, restés en place après la despinalisation, représenteraient une source d'innervation quantitativement inadéquate pour assurer, à elle seule, le rôle inducteur, normalement joué par la moelle épinière dans la régénération caudale.

Nous avons alors cherché à substituer à la moelle une masse suffisante de tissu cérébral. Nous avons transplanté du télencéphale dans le canal rachidien, préalablement évidé. Pour cela, on dénude la partie rostrale du crâne et on ouvre la voûte cranienne, juste au-dessus du lobe olfactif gauche. Une large portion du télencéphale gauche est ensuite prélevée et greffée sur le moignon caudal despinalisé du même animal (fig. 1*b*).

Nous avons effectué vingt et une opérations de cette sorte. Sur les dix-sept cas examinés histologiquement, nous avons pu retrouver des fragments de cerveau greffé, dans sept moignons de queue despinalisée. L'état de ces transplants cérébraux, deux mois après l'intervention, est excellent. Leur taille varie entre 600 et 1.800 μ. Les cellules et les fibres semblent saines. Il y a de nombreuses mitoses et des faisceaux de fibres nerveuses sortent des greffons en direction du niveau d'amputation. Le plus souvent, la greffe télencéphalique est libre dans le mésenchyme caudal et forme un ou deux amas sphériques ou plurilobés contenant des cellules nerveuses et des fibres. Dans un cas, le transplant est enkysté et dans un autre il est dissocié en plusieurs fragments de taille inégale. Même dans ces cas, on n'observe aucune altération cytologique apparente (Planche 2, figs. 13, 14, 15).

Malgré l'état sain des greffes cérébrales, on n'assiste jamais à une induction d'axe caudal, à moins qu'une régénération consécutive de la moelle épinière du moignon ait eu lieu. Le tissu télencéphalique s'avère donc incapable de suppléer à l'absence de la moelle épinière dans la régénération caudale.

5. *Introduction des deux nerfs sciatiques dans la queue despinalisée*

Si la moelle épinière agit sur la régénération caudale par l'importance quantitative de ses fibres nerveuses, on doit, en principe, obtenir un résultat identique en remplaçant les fibres de la moelle par d'autres provenant, par exemple, des nerfs sciatiques. Cette condition est réalisée quand on dévie un nerf sciatique dans le territoire caudal. Mais, on pourrait objecter qu'un seul nerf sciatique n'apporte pas suffisamment de fibres nerveuses pour suppléer avec succès à l'absence totale de la moelle épinière. Pour répondre à cette objection, nous avons réalisé une série d'artifices expérimentaux.

(*a*) Dans un premier temps, les pattes postérieures seules sont irradiées avec une dose totale de rayons X de 1.000 r. environ (Tube Westinghouse, 70 kV., 19 mA., distance du foyer 43 cm., dose 90 r./minute). Cette dose suffit généralement pour inhiber la régénération des pattes (voir Luther, 1948, et nos témoins) (fig. 1*c*).

(*b*) Après un laps de temps de sept jours, pour permettre aux animaux irradiés de surmonter la période inflammatoire initiale, on ampute la queue à une distance de 5 mm. du cloaque et on despinalise le moignon sur une longueur de 3 mm. On pratique ensuite deux profondes gouttières sur les flancs de ce moignon despinalisé et on y insère les deux pattes postérieures irradiées, en dénudant leurs faces internes et en exposant les nerfs sciatiques sur presque toute leur longueur (fig. 1*d*). Les deux pattes, amputées au niveau du zeugopode, et le moignon caudal sont maintenus ensemble, pendant vingt-quatre heures, par une ligature au fil de nylon; quand la ligature est enlevée, pattes et queue forment un tout et les deux nerfs sciatiques pénètrent dans les tissus de la queue despinalisée.

La mortalité fut assez élevée. Sur vingt-sept animaux ainsi opérés, onze seulement ont survécu assez longtemps pour être inclus dans cette étude. Deux d'entre eux ont présenté une régénération de la moelle épinière, qui eut pour conséquence la reconstitution de l'axe caudal. Tous les autres, chez lesquels la présence des deux nerfs sciatiques réunis a été vérifiée histologiquement, ont été incapables d'une véritable morphogenèse. Nous n'avons observé qu'une prolifération du conjonctif des nageoires, dont la structure est analogue à celle que nous avons constatée lors d'une simple despinalisation de la queue (Planche 2, fig. 16). Seul l'aspect extérieur de ces régénérats caudiformes est différent: le tissu des nageoires s'étend sur les moignons des pattes irradiées et forme des lames disposées sur un plan frontal ou oblique (fig. 3*a*, *b*, dans le texte; Planche 1, figs. 9, 10). L'ensemble donne au train postérieur de l'animal l'apparence d'une 'queue d'avion'.

Cette prolifération préférentielle du tissu des nageoires au niveau des membres postérieurs irradiés, remplacée par une régénération normale des pattes chez les témoins non irradiés, doit être en rapport avec la présence, au voisinage, d'une riche innervation. Chaque fois qu'on met en contact du tissu caudal avec un nerf, on observe une prolifération semblable. Que ce soit la greffe de peau de queue au niveau de la patte ou la déviation d'un nerf sciatique vers le flanc de la queue (Guyénot & Ponse, 1930; Bovet, 1930), le résultat est toujours le même: formation d'un organe caudiforme du type 'nageoire', dépourvu de squelette et de musculature.

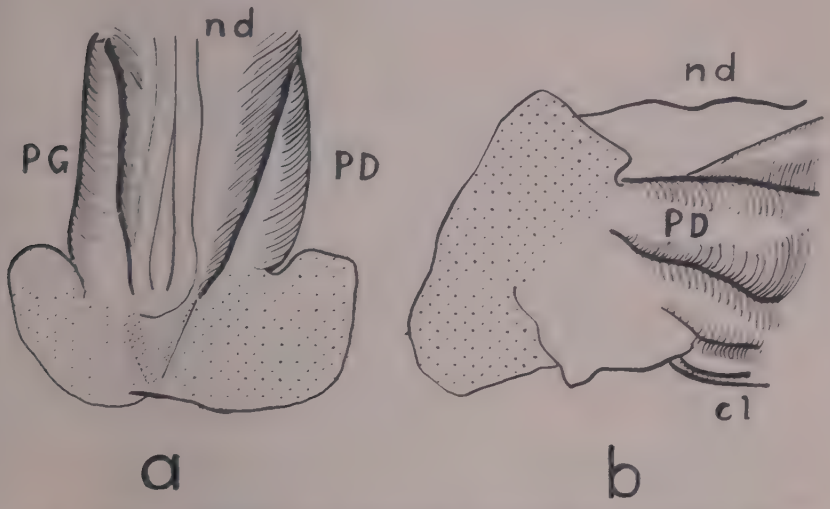

FIG. 3. Dessins à la chambre claire des structures régénérées après irradiation des pattes postérieures et leur incorporation dans un moignon caudal despinalisé. *a*, vue dorsale (cf. Planche 1, fig. 10) d'un cas avec régénérats du type nageoire sur un plan frontal. *b*, un autre cas vu de profil (cf. Planche 1, fig. 9). Le régénérat du type nageoire est ici oblique. *nd*, nageoire dorsale. *PD–PG*, pattes droite–gauche. *cl*, cloaque. En pointillé, le régénérat.

Nous pensons que la régénération normale des nageoires doit être favorisée, sinon absolument conditionnée, par le système nerveux périphérique, les fibres nerveuses qui, nous l'avons vu, pénètrent profondément dans de tels régénérats.

Conclusion. L'ensemble de ces expériences montre que l'absence locale de la moelle épinière arrête la prolifération des tissus de la queue, à l'exception de la nageoire, et empêche la formation d'un régénérat caudal typique. Tout essai de substituer à la moelle épinière d'autres éléments nerveux s'est soldé par un échec. Les ganglions rachidiens, le cerveau (télencéphale) et les deux nerfs sciatiques réunis ont été incapables d'induire quoi que ce soit dans une queue despinalisée. L'action de la moelle épinière dans la régénération caudale semble spécifique.

II. *Spécificité des zones du territoire 'queue'*

1. *Déviation de la moelle épinière*

Quand on fait la despinalisation du moignon caudal, il est facile de dévier et de faire sortir vers l'extérieur l'extrémité de la moelle épinière restante (fig. 1*e*). De cette façon, d'une part on empêche toute pénétration tardive de la moelle dans le canal rachidien évidé et, d'autre part, on crée un nouveau centre de prolifération et de croissance régénérative, par un mécanisme analogue à celui qu'amorce la déviation d'un nerf dans le territoire 'patte'. Le résultat en est l'apparition d'une queue latérale surnuméraire.

Nous avons obtenu, en tout, vingt-six de ces formations que nous avons réparties en deux groupes: queues latérales (22 cas) et queues dorso-latérales (4 cas).

(*a*) Par son aspect extérieur une queue latérale rappelle un axe caudal, entouré de peau, sans trace de nageoire. C'est une excroissance cylindrique, plus ou moins allongée, dont l'extrémité se termine en pointe (Planche 1, fig. 8; Planche 3, fig. 18). L'examen histologique confirme ces observations. En coupe transversale on voit, au centre, l'axe cartilagineux surmonté de la moelle régénérée. Des muscles métamériques sont disposés en cercle autour des organes axiaux. Il n'y a pas trace de nageoire (Planche 3, fig. 24). Sur des coupes frontales ou sagittales, on voit la métamérisation du cartilage vertébral, l'absence totale de restes de chorde dorsale à son intérieur, ce qui indique une néoformation, et, enfin, la moelle épinière du régénérat qui n'est que la continuité de la moelle ancienne (Planche 3, fig. 21). Dans quelques cas, on voit même des ganglions spinaux régénérés.

Partout la queue latérale prend naissance en plein dans l'axe de la queue normale, à mi-chemin entre les nageoires, dorsale et ventrale.

(*b*) Les queues dorso-latérales résultent d'une déviation de la moelle en direction légèrement dorsale. En réalité, toutes les opérations ont été effectuées de la même façon. L'ouverture latérale un peu plus étendue a permis un déplacement du bourgeon vers le dos. Il se forme un axe caudal typique, analogue à celui que nous venons de décrire, surmonté d'une hémi-nageoire caudale surnuméraire (Planche 3, figs. 19, 20).

La fig. 4 représente l'évolution d'un tel régénérat. De même, une coupe, passant à travers la queue despinalisée *in situ* et la queue surnuméraire latérale, montre bien les rapports anatomiques et la structure histologique de ces formations (Planche 3, fig. 23).

Pour des raisons techniques, nous n'avons pas pratiqué la déviation ventro-latérale de la moelle; le point à atteindre, c'est-à-dire la limite entre la zone axiale de la queue et la nageoire ventrale, étant assez éloignée de l'emplacement du canal rachidien.

Tandis que, dans toutes sortes d'opérations intéressant le territoire caudal, la nageoire est la formation la plus fréquemment réalisée, l'apparition d'une

hémi-nageoire n'a été observée ici que dans quatre cas sur vingt-six. L'axe caudal, partie médiane de la queue, semble donc posséder des potentialités morphogènes limitées. A ce niveau, l'induction exercée par la moelle épinière provoque uniquement la formation d'un axe caudal surnuméraire.

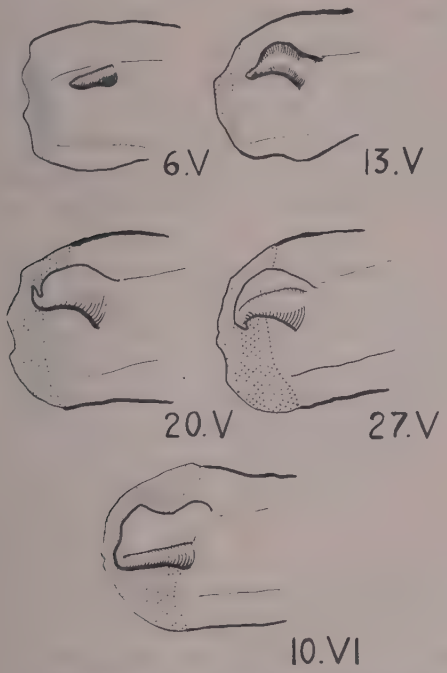

FIG. 4. Évolution d'une queue despinalisée avec déviation dorso-latérale de la moelle restante. En pointillé: régénérat de nageoire, sans axe musculo-squelettique. Le régénérat dorso-latéral contient des vertèbres et de la musculature et développe une nageoire surnuméraire, mais seulement du côté dorsal.

2. *Greffe de moelle dans la nageoire caudale*

Si l'axe caudal a des potentialités morphogènes limitées, qu'en est-il de la région des nageoires? Pour répondre à cette question, nous avons pratiqué l'opération suivante:

Après despinalisation du moignon caudal (fig. 1*e*), le fragment de la moelle épinière, sorti du canal rachidien, est coupé en deux (fig. 1*f*) et chaque portion est introduite dans les nageoires, dorsale et ventrale, du moignon, selon une technique inspirée par celle de Weiss (1950). Les deux fragments de moelle sont disposés parallèlement à l'axe caudal, mais aussi éloignés de lui que possible (fig. 1*g*). On obtient ainsi des queues, dont l'axe est despinalisé et dont les deux

nageoires sont munies chacune d'un fragment de moelle épinière, qui devrait créer à son niveau des centres de prolifération, d'autant plus facilement que la despinalisation de l'axe, en inhibant sa propre régénération, laisse le champ libre de toute concurrence. Nous avons opéré de la sorte au total vingt-cinq larves (dix de *Salamandra* et quinze de *Triturus cristatus*) dont vingt-trois ont pu être conservées jusqu'à la métamorphose. Malgré les différentes orientations des greffons de moelle (antéro-antérieure, antéro-postérieure, dorso-dorsale, dorso-ventrale) nous n'avons jamais observé la moindre induction d'axe caudal.

Dans un cas, il s'est formé au niveau de la greffe supérieure une protubérance digitée. Mais l'examen histologique a révélé que sa structure était purement conjonctive, sans trace d'organisation à son intérieur. Nous avons examiné histologiquement tous ces moignons de queue. Les greffons de moelle, qui étaient visibles par transparence chez la larve, ont subi le contre-coup de l'involution générale des nageoires caudales, involution consécutive à la métamorphose. Sur les coupes, on les retrouve comme des amas cellulaires allongés présentant les signes d'une dégénérescence pigmentaire prononcée. En face de la moitié ventrale (motrice) des transplants de moelle, le tissu conjonctif lâche de la nageoire présente une certaine condensation fibrillaire, d'ailleurs peu importante, visible surtout par une couleur bleue marquée, après coloration au Mallory–Azan. Mais il n'y a jamais de véritable organogenèse: pas de cartilage, pas de muscles métamériques, pas de ganglions. On a l'impression que la moelle épinière greffée a exercé au maximum son action inductrice, mais que le terrain lui était peu favorable.

Conclusion. Les déviations dorsales et dorso-latérales de la moelle épinière au niveau de l'axe caudal et la greffe de moelle épinière dans les nageoires, dorsale et ventrale, montrent que toutes les parties de la queue ne réagissent pas de la même façon à l'action inductrice de la moelle.

On peut distinguer, selon la réponse morphogénétique, deux zones dans le territoire 'queue'. Une zone centrale, l'axe caudal, qui régénère, sous l'impulsion de la moelle épinière, la colonne vertébrale et la musculature métamérique, et une zone périphérique, les nageoires, qui peuvent se reconstituer et s'allonger même sans la moelle (mais probablement pas en l'absence de tout élément nerveux).

A la spécificité inductrice de la moelle épinière correspond une spécificité organoformatrice des zones du territoire 'queue'.

Addendum. Phénomènes d'histotropisme. Au cours de ces expériences, nous avons eu l'occasion d'observer des phénomènes d'histotropisme, c'est-à-dire d'affinité entre éléments tissulaires appartenant au système nerveux.

(*a*) Les fragments de cerveau greffés montrent une certaine affinité entre eux. Ils sont souvent réunis par des ponts de fibres nerveuses. Par contre, on ne constate aucune attraction réciproque entre transplants de cerveau et moelle épinière. Cette dernière semble polarisée en direction postérieure; elle peut passer au cours de sa croissance régénérative à côté d'une greffe de cerveau sans

qu'il s'établisse la moindre connexion entre ces deux parties du système nerveux central.

(*b*) La situation est tout autre entre moelle épinière et ganglions nerveux qui présentent une affinité qui peut, parfois, prendre des proportions extraordinaires. Dans les moignons despinalisés, les ganglions persistent isolés. Dans un cas, l'un d'entre eux s'est mis en rapport avec la moelle épinière régénérée de la queue latérale. Le chemin parcouru par les fibres reliant ce ganglion à la moelle est long (Planche 2, fig. 17) et s'élève à environ 4.920 μ. Ce pont fibrillaire est jalonné de deux condensations cellulaires (ganglions de relais ?). Il faut remarquer que l'orientation des fibres nerveuses sortant du ganglion ancien a été inversée pour que ces dernières puissent rencontrer, après un long parcours, la nouvelle moelle épinière.

DISCUSSION

Trois problèmes majeurs se posent lors de l'étude de la régénération caudale des Urodèles:

(*a*) Le mécanisme d'apparition et de différenciation du régénérat.

(*b*) Le rôle inducteur joué par le système nerveux.

(*c*) Les potentialités morphogènes des tissus induits.

(*a*) *Apparition et différenciation du régénérat*

La modalité de la mise en place des ébauches, au cours de la régénération caudale, est encore un sujet de controverse. Selon une première hypothèse (Nicholas, 1955), chaque partie du moignon produirait, par croissance continue, les tissus correspondants du régénérat, selon le principe *similia similibus*. Si un élément de la queue vient à manquer, la partie homologue du régénérat fera obligatoirement défaut. L'intégrité de la surface d'amputation est la condition *sine qua non* d'une régénération caudale complète.

Une seconde hypothèse postule la formation préalable d'un blastème indifférencié pluripotent, à partir duquel se différencieraient les tissus du régénérat (S. Holtzer, 1956).

Il y a, enfin, une position intermédiaire selon laquelle, outre un blastème mésodermique aux potentialités morphogènes limitées, il y aurait des parties formées directement par leurs équivalents contenus dans le moignon (Newth, 1958; H. Holtzer, 1959).

La première de ces hypothèses semble maintenant, du moins dans son expression la plus stricte, infirmée par une série d'expériences et d'observations. La chorde dorsale, dont il reste dans le moignon d'importantes portions, ne se prolonge jamais dans le régénérat (Barfurth, 1891; H. Holtzer, S. Holtzer & Avery, 1955). Le squelette vertébral cartilagineux peut se former en l'absence du squelette ancien (H. Holtzer, 1959). Les ganglions spinaux et les nerfs se reconstituent *de novo* à partir de la moelle épinière (Stefanelli & Capriata, 1944).

L'hypothèse du blastème a été formulée par analogie avec la régénération de la patte. Elle aussi ne semble pas entièrement satisfaisante.

La moelle épinière du régénérat dérive directement de celle du moignon par extension et prolifération cellulaire. Certaines parties, comme les nageoires, se reconstituent de façon indépendante et ne se forment pas aux dépens du blastème mésodermique de l'axe caudal (Newth, 1958 et nos propres expériences).

On est donc amené à considérer la troisième hypothèse comme la plus conforme aux faits.

(*b*) *Le rôle du système nerveux*

L'action inductrice de la moelle épinière sur le régénérat caudal, implicitement reconnue par Godlewski (1928) et Okada (1938), a été démontrée par H. Holtzer (1955) et ses collaborateurs; sans moelle épinière, il n'y a pas de régénération d'axe caudal.

La validité de cette proposition semble contestée par les expériences de Liosner et Woronzowa (1937), de Trampusch (1958) et de Glade (1957). H. Holtzer, dans une publication récente (1959) réfute ces arguments. Nous pensons que les expériences rapportées ici confirment pleinement les vues de Holtzer.

Il faut souligner que les auteurs qui prétendent avoir obtenu des régénérats complets sans moelle épinière ont toujours transplanté certains tissus de la queue en position hétérotopique, le plus souvent sur la patte amputée. Or, nous avons vu les aléas que présente la transplantation hétérotopique. Il est peu probable que le fait de greffer ces tissus à un autre endroit qu'à leur emplacement normal leur confère des qualités morphogènes qu'ils sont incapables de montrer *in situ*. Il est plus plausible d'admettre, soit une inclusion fortuite de fragments de moelle dans les greffons, ce qui fut effectivement le cas dans les expériences de Trampusch, soit une homologation erronée des éléments squelettiques du régénérat. La succession du carpien, du métacarpien et des phalanges d'un seul doigt peut simuler l'aspect des cartilages métamériques d'une colonne vertébrale en voie de régénération.

Si la moelle épinière est indispensable à la régénération de l'axe caudal, quelle est la nature de son action?

On pourrait envisager une action non spécifique de la moelle en tant que tissu nerveux émettant des fibres. Cette hypothèse nous a été suggérée par les observations de Guyénot et de son école et par celles, plus récentes, de Singer (1954) sur la régénération de la patte. Dans ce cas, le moignon doit contenir un certain nombre de fibres nerveuses pour régénérer. Au-dessous d'un seuil inférieur quantitatif, le blastème régénérateur ne se constitue plus. L'action du système nerveux est, dans ce cas, banale, non spécifique, puisqu'on peut obtenir, soit, avec le même nerf, les structures les plus diverses, selon le territoire excité (Guyénot & Schotté, 1926; Bovet, 1930), soit la formation d'une patte grâce à une innervation différente, par exemple, celle de la queue. L'expérience réciproque, greffe de tissu caudal sur le membre amputé, ne donne que des organes

caudiformes (Guyénot & Ponse, 1930). Ce dernier résultat pourrait être attribué, *a priori*, non pas à l'incapacité fondamentale du système nerveux périphérique d'induire un blastème caudal, mais à l'insuffisance quantitative de l'innervation fournie par une seule patte. Afin d'éliminer cette dernière objection, nous avons introduit dans la queue despinalisée *in situ* l'innervation des deux membres postérieurs, eux-mêmes préalablement irradiés, pour empêcher leur régénération concurrentielle. Nous savons que l'augmentation du nombre des fibres nerveuses au-dessus de la normale peut stimuler la croissance de tissus habituellement réfractaires (Singer, 1954). Mais, malgré la présence de toutes ces conditions favorables, l'apparition d'un axe caudal a toujours fait défaut.

Si les fibres nerveuses seules ne peuvent induire la régénération de la queue, on pourrait supposer que la moelle épinière partage au moins cette propriété avec les autres parties du système nerveux central, contenant des neurones. En réalité, il n'en est rien. Nous avons montré que la présence des ganglions rachidiens dans le moignon despinalisé ne suffit pas à assurer la régénération complète de la queue. Au contraire, dans la patte, l'induction d'un blastème peut se faire par un ganglion spinal greffé dans le moignon (Kamrin & Singer, 1959).

Le cerveau pourrait-il remplacer la moelle épinière dans son action? Nous avons greffé le télencéphale dans la queue despinalisée. Malgré le bon état des transplants qui présentaient des cinèses et émettaient des fibres vers la surface d'amputation, il n'y a jamais eu, à leur voisinage, formation d'axe caudal.

Une certaine polarisation de la moelle épinière serait-elle responsable de son effet inducteur? C'est à dessein que nous avons utilisé pour nos greffes les lobes olfactifs, fragments du système nerveux central également polarisés. Le résultat, nous l'avons vu, est négatif. D'ailleurs, l'induction par un fragment de moelle se manifeste aux deux extrémités, s'il y a deux surfaces d'amputation (S. Holtzer, 1956).

En définitive, l'induction de l'axe caudal est l'apanage exclusif de la moelle épinière. C'est la moitié ventrale, motrice, de la moelle qui en est seule responsable (H. Holtzer, 1959). Le lobe olfactif du cerveau et les ganglions rachidiens, centres sensitifs, ainsi que les nerfs périphériques, sont incapables de se substituer à l'action de la moelle, qui semble donc spécifique.

(*c*) *Les potentialités morphogènes des zones du territoire caudal*

Si le territoire 'queue' était isodyname sur toute son étendue, la moelle épinière, qui en est l'inducteur spécifique, devrait pouvoir induire partout un régénérat caudal complet. Or, nous avons vu que ce n'est pas le cas. Avec les éléments mésodermiques du milieu de la queue, la moelle détermine la formation d'un axe caudal surnuméraire, et ceci dans des conditions expérimentales variées: déviation (Kiortsis & Droin, 1958; H. Holtzer, 1959), greffe (S. Holtzer, 1956) ou stimulation après irradiation (Brunst, 1950, 1952). La même moelle, greffée seule dans la région des nageoires, se montre incapable d'induire des structures semblables (Overton, 1950, S. Holtzer, 1956). Enfin, si l'extrémité de la moelle

déviée aboutit à la limite entre les deux régions, on observe l'apparition d'organes mixtes, véritables chimères de nageoire et de l'axe caudal.

On pourrait objecter que les formations mixtes, obtenues lors d'une déviation dorso-latérale de la moelle, résultent simplement d'un élargissement de la plaie en direction dorsale. Ce serait un argument en faveur de la théorie qui admet que seules les parties présentes à la surface d'amputation régénèrent. Toutefois dans les expériences de Luther (1948) et de Trampusch (1958), on trouve des cas où l'axe caudal, ayant été entouré d'un manchon de peau neutre, a régénéré une queue avec des nageoires, qui sont sans rapport de continuité avec les anciennes et plus étendues que la surface de section. Manifestement, dans le manchon, on a enveloppé avec l'axe des éléments cellulaires, appartenant à la zone des nageoires. Par la suite, ces éléments ont formé des nageoires aplaties qui ne sont pas limitées par la surface circulaire d'amputation.

La nature des organes caudiformes formés après déviation d'un nerf au sein du territoire 'queue' (Guyénot & Schotté, 1926; Bovet, 1930) ou après transplantation de tissus de ce territoire au niveau de la patte (Guyénot & Ponse, 1930) est élucidée. Ces formations résultent d'une part de processus réparateurs du squelette et de la musculature lésés, d'autre part d'une prolifération régénérative du flanc du territoire 'queue'. On ne pourrait les considérer comme des organes hypotypiques, dus à une innervation insuffisante, puisque l'énorme apport de fibres nerveuses des deux nerfs sciatiques réunis n'ajoute rien à leur structure, purement conjonctive. D'ailleurs, si les régénérats du type 'nageoire' résultaient d'une diminution progressive de l'innervation, on devrait s'attendre à voir les queues latérales surnuméraires se terminer par un aplatissement; or, elles sont toujours terminées en pointe.

L'ensemble de ces faits indique que le territoire 'queue' se divise en deux zones, à potentialités morphogènes distinctes. Chacune de ces zones a une origine embryologique différente: l'axe caudal est purement mésodermique, tandis que la nageoire dérive des crêtes neurales (Newth, 1958; Bijtel, 1958). Les éléments de ces dernières sont impropres à la chondrification et à la squelettogenèse (E. W. Okada, 1955).

En définitive, la zone de l'axe caudal forme, sous l'induction de la moelle, un blastème qui se différencie par la suite en cartilages et muscles métamérisés. La zone de la nageoire régénère, par extension ou par blastème, une lame de conjonctif. C'est la croissance simultanée et harmonieuse des deux zones qui produit un régénérat normal de queue.

RÉSUMÉ

1. L'autogreffe de tranches de queue sans moelle sur le dos et la despinalisation du moignon caudal *in situ* ne sont suivis d'aucune néoformation squelettique ou musculaire. Il apparaît, à la surface d'amputation, un régénérat conjonctif, du type 'nageoire'.

2. Seule la moelle épinière est capable d'induire l'axe caudal. Ni les deux nerfs sciatiques, ni les ganglions rachidiens restés en place, ni le télencéphale greffé, ne sont en mesure de se substituer à la moelle, dans son action inductrice. Son effet sur la régénération caudale semble spécifique.

3. La déviation latérale de la moelle produit des queues surnuméraires composées, soit exclusivement d'axe caudal, soit, si la déviation est faite en direction dorsale, d'un axe surmonté d'une nageoire. La greffe de segments de moelle dans les tissus mêmes des nageoires n'induit pas, à ce niveau, la formation de fragments squelettiques ou musculaires. Il faut donc, pour la régénération complète de la queue: (*a*) la moelle, (*b*) des éléments mésodermiques de l'axe, et (*c*) des cellules de la région des nageoires, dérivées de la crête neurale.

4. Le territoire 'queue' se compose de deux zones ayant des origines et des potentialités morphogènes distinctes: une zone centrale, produisant l'axe musculo-squelettique et une zone périphérique, formant des nageoires.

SUMMARY

The role played by the spinal cord and other tissues in the regeneration of the tail was studied in larval salamanders.

1. Transverse tail-slices, with or without spinal cord, grafted to the back may undergo a reduction in size, or remain stationary, or even regenerate new structures. A more marked reduction was observed in the despinalized grafts.

2. After partial amputation of the tail, a despinalization of the stump was performed without injuring the other tissues. In all cases where the absence of the spinal cord was proved histologically, no regeneration of the mesodermal axis occurred. Only a fin-like regenerate without muscles or skeleton appeared at the amputation surface.

3. Spinal ganglia or brain (telencephalon), substituted for the spinal cord of the tail, were unable to replace it in its inductive action. An increase of the nerve supply, by introducing the two sciatic nerves into the despinalized tail-stump, also had no effect. It seems that the inductive action exerted by the spinal cord on tail regeneration is specific.

4. The lateral deflexion of the spinal cord was enough to induce a supernumerary tail. If the deflexion was done dorsolaterally, instead of laterally, the induced supernumerary tail formed a half-fin too. Segments of the spinal cord, grafted dorsally or ventrally into the tail-fin, at some distance from the main axis, did not induce any muscular or skeletal structures.

5. It is believed that the organ district of the tail contains at least two zones of different morphogenetic potencies: one mesodermal, which regenerates the main axis of the organ, under the inductive influence of the spinal cord, and one, derived from the neural crest, which produces, even in the absence of the spinal cord, a fin-like structure.

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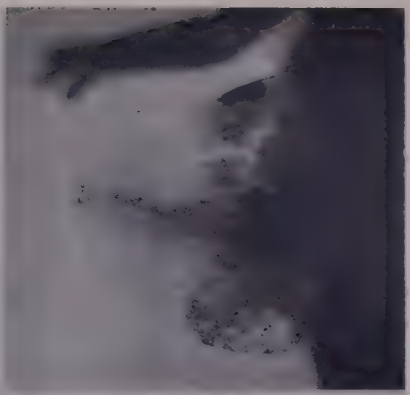

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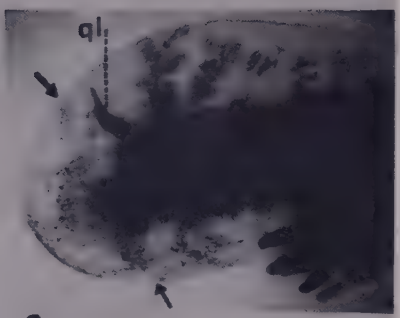

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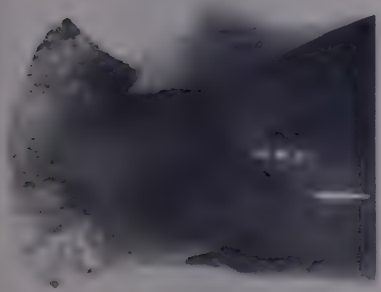

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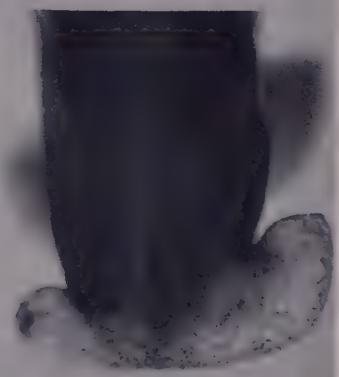

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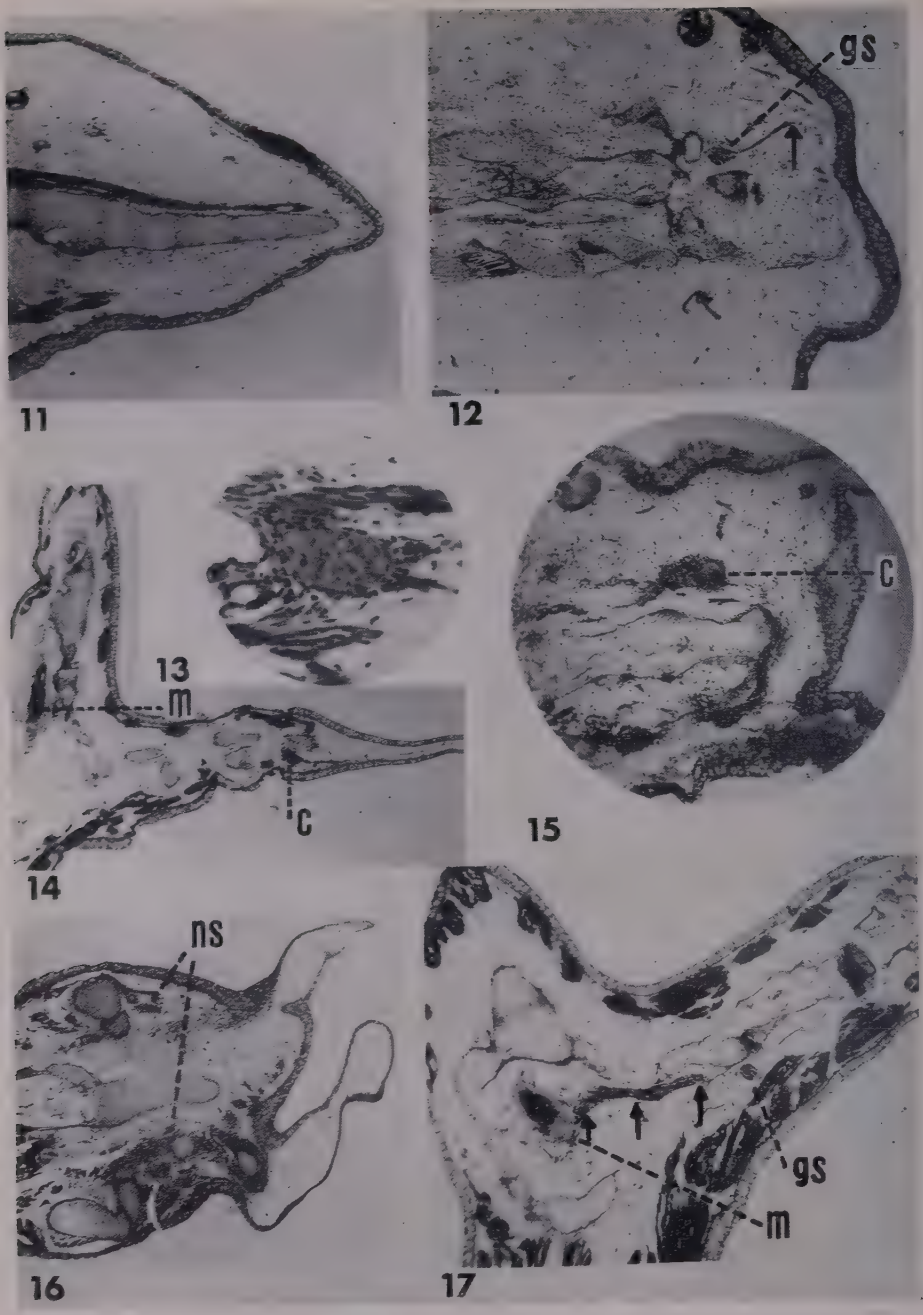
V. KIORTSIS *et* A. DROIN

Planche 1



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EXPLICATION DES PLANCHES

PLANCHE 1

Fig. 5. Tranche de queue despinalisée, greffée sur le dos. Régénérat sans squelette, sans muscles.

Fig. 6. Tranche de queue normale (avec moelle) greffée sur le dos. Axe musculo-squelettique visible par transparence dans le régénérat.

Fig. 7. Queue despinalisée *in situ*. Régénérat du type 'nageoire', bien vascularisé, sans axe musculo-squelettique. Les flèches indiquent le niveau d'amputation.

Fig. 8. Despinalisation de la queue *in situ* et déviation latérale de la moelle épinière. Régénérat du type 'nageoire' au niveau d'amputation (flèches). *ql*, petite queue latérale, cylindrocônique, sans nageoires, née sur déviation.

Figs. 9 et 10. Structures régénérées après irradiation des pattes postérieures, amputation et incorporation dans le moignon caudal, préalablement despinalisé (cf. fig. 3 dans le texte).

Fig. 9. Un cas vu de profil. Régénérat oblique de type 'nageoire'.

Fig. 10. Un autre cas vu de dos. Régénérat frontal de type nageoire.

Figs. 5 à 9, photographies non retouchées.

PLANCHE 2

Fig. 11. Coupe sagittale d'un régénérat normal de queue. Les restes de la chorde dorsale permettent de reconnaître les anciens tissus.

Fig. 12. Coupe sagittale d'un moignon de queue despinalisée. Persistance d'un ganglion spinal (*gs*) d'où partent des fibres nerveuses (flèche) pénétrant dans le régénérat caudiforme.

Fig. 13. État sain d'un transplant de cerveau greffé dans une queue despinalisée: fibres et neurones.

Fig. 14. Coupe frontale d'un moignon de queue despinalisée avec greffe de cerveau (*c*) et d'une queue latérale formée après déviation de la moelle épinière (*m*). Malgré la transplantation du cerveau qui envoie des fibres vers la surface d'amputation (visibles sur la photo), le moignon caudal n'a formé qu'un régénérat du type 'nageoire'. Par contre la moelle déviée a induit un axe vertébral cartilagineux et des muscles métamériques.

Fig. 15. Coupe sagittale d'un moignon caudal despinalisé avec un gros transplant de cerveau (*c*).

Fig. 16. Coupe frontale d'un moignon de queue despinalisé auquel sont incorporées les deux pattes postérieures préalablement irradiées. Les deux nerfs sciatiques (*ns*), droit et gauche, pénètrent dans le territoire 'queue' mais ne peuvent se substituer à l'action inductrice de la moelle épinière.

Fig. 17. Attraction exercée par la moelle épinière déviée (*m*) sur les fibres nerveuses d'un ganglion spinal (*gs*) resté isolé dans une queue despinalisée. Tractus nerveux anormalement long (flèches).

Figs. 11 à 17: Microphotographies non retouchées.

PLANCHE 3

FIGS. 18, 19, et 20. Vues dorsales des trains postérieurs de trois larves de salamandre avec queues surnuméraires (*ql*) nées sur déviation latérale (fig. 18) ou dorso-latérale (figs. 19, 20) de la moelle épinière.

FIG. 18. Régénérat cylindrique avec axe squelettique et musculature sans trace de nageoire.

FIG. 19. Régénérat avec nageoire dorsale sans communication apparente avec la nageoire du moignon.

FIG. 20. Confluence proximale des deux nageoires dorsales.

FIG. 21. Coupe frontale. Horizontalement, régénérat d'un moignon caudal despinalisé: aucun élément squelettique ou musculaire. Verticalement: queue latérale née sur la déviation de la moelle épinière. Régénérat caudal complet avec squelette en voie de segmentation, moelle épinière et musculature métamérique.

FIG. 22. Faisceau de fibres nerveuses pénétrant profondément dans un régénérat sans axe d'une queue despinalisée (imprégnation argentique selon Bodian).

FIG. 23. Coupe transversale d'une queue despinalisée comprenant, en haut et à gauche, les structures régénérées après déviation dorso-latérale de la moelle épinière. *ql*, queue latérale avec cartilage vertébral, moelle et musculature métamérique. *n*, nageoire dorsale de la queue. *n'*, nageoire dorsale surnuméraire de la queue latérale.

FIG. 24. Coupe transversale d'une queue latérale cylindrique sans nageoires.

FIG. 25. Coupe frontale d'un moignon caudal despinalisé. On distingue la frontière fibreuse entre les anciens tissus et le régénérat mésenchymateux. La flèche indique le cartilage de réparation formé au niveau de l'amputation.

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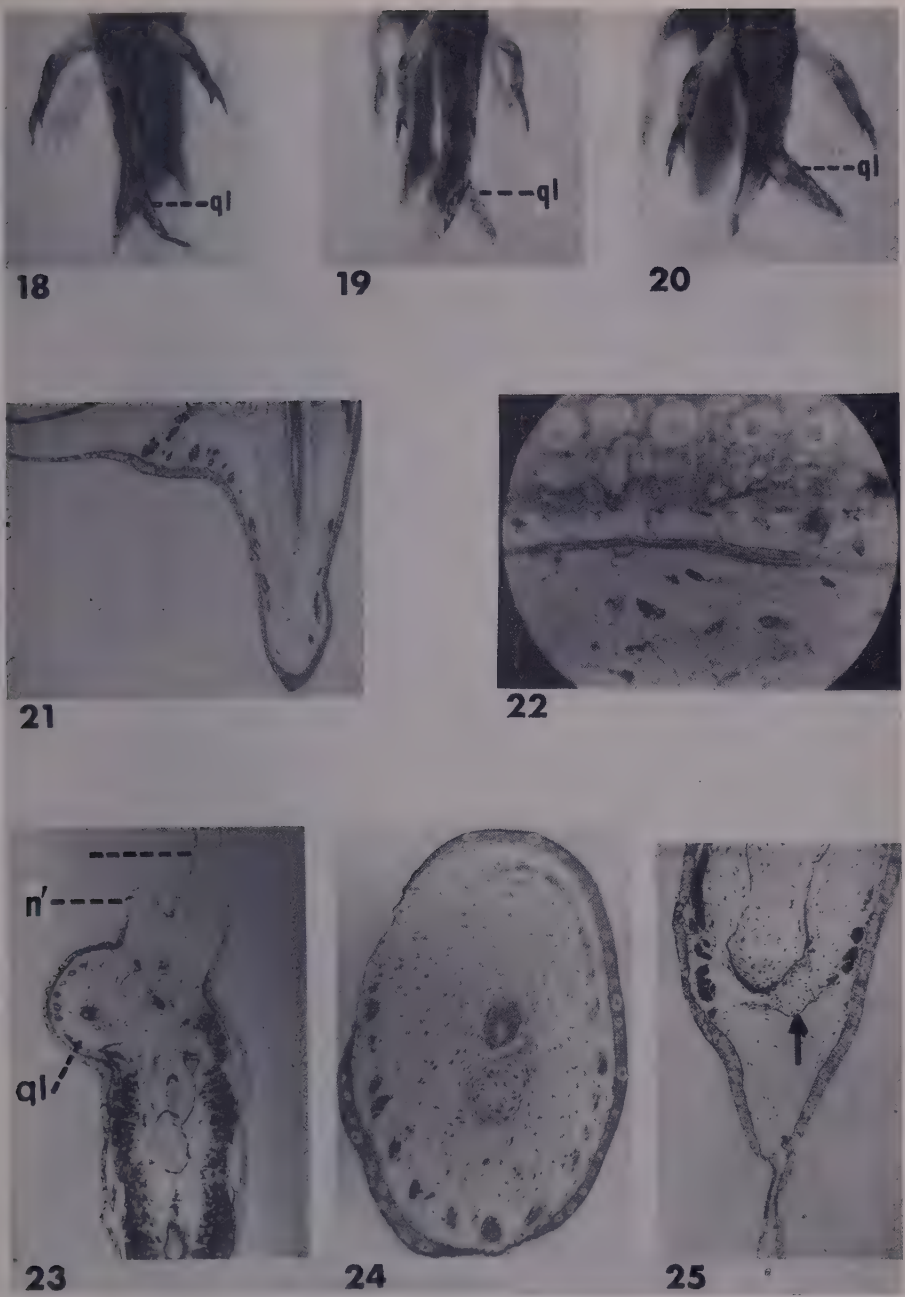

V. KIORTSIS *et* A. DROIN

Planche 3

The Effect of Delayed Brain Extirpation and Replacement on Caudal Regeneration in *Nereis diversicolor*

by R. B. CLARK *and* S. M. EVANS[1]

From the Department of Zoology, University of Bristol

INTRODUCTION

THE normal regeneration of amputated posterior segments in the polychaete *Nereis diversicolor* is possible only if the supra-oesophageal ganglion is intact (Clark & Bonney, 1960). Worms fail to regenerate if the ganglion is extirpated before the segments are amputated, but if the ganglion is removed 3 days after amputation of the segments, regeneration proceeds, though at a slower rate than in cerebrate animals. Histological examination of the supra-oesophageal ganglion of regenerating worms at various times after the loss of the posterior segments reveals an increase in cerebral neurosecretory activity within a few hours of the loss of segments. This increased production of neurosecretory material is consistent with the view that cerebral hormones initiate or otherwise promote regeneration, a point we have attempted to confirm in the present paper, and in this respect the Nereidae are comparable with the lumbricid oligochaetes (Hubl, 1956) and nephtyid polychaetes (Clark & Clark, 1959).

In both lumbricids and nephtyids the changes in the appearance of the neurosecretory cells in the supra-oesophageal ganglion are sudden and unmistakable. In *N. diversicolor* they are much less obvious, suggesting, among other possibilities, that the ganglionic influence upon regeneration is exerted more gradually than in the other worms. In order to investigate this we have studied the effect of delayed brain extirpation, and also of reimplanting brains or injecting minced brain into decerebrate worms at various times after the loss of the posterior segments.

The rate at which *N. diversicolor* regenerates is extremely variable. Apart from temperature, sex, the number of segments lost, &c., all of which are known to influence the regeneration rate and which can be controlled, there remains a considerable individual variation in the number of new segments proliferated within a given period after caudal amputation. In the design of the experiments and the assessment of the results we have therefore attached greater importance

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to the type of regeneration that occurs than to the actual number of new segments formed.

Following Stéphan-Dubois (1956) we recognize the following stages in the regeneration of this worm:

1. Wound-healing, when only a cap of epidermal tissue forms over the wound.
2. Pygidium formation, in which a small knob-like pygidium, generally with a pair of anal cirri, is formed.
3. Vascularized pygidium formation, in which the dorsal blood-vessel, generally with lateral side-branches, extends into the new pygidium.
4. Segment proliferation, indicated initially by the appearance of ventro-lateral parapodial rudiments on the new pygidium.

Under the conditions of our experiments, regeneration may be arrested at any of these stages.

MATERIALS AND METHODS

The *N. diversicolor* used in these experiments were collected at Newquay (Cornwall), Portishead (Somerset), and Cullercoats (Northumberland) during the autumn and winter. Stéphan-Dubois (1956) claims that worms approaching sexual maturity regenerate more slowly than immature specimens, but we have not found this to be so, and worms in various stages of sexual maturity have been used in our experiments. The general procedure has been similar to that described previously (Clark & Bonney, 1960), except that MS/222 (Sandoz Products) has been used in place of isotonic magnesium chloride solution as an anaesthetic. Using a 0·05 per cent. solution of MS/222 in sea-water, medium-sized worms are completely narcotized within 5–7 minutes and recover equally rapidly on being returned to fresh sea-water. Loss of blood following removal of the brain was prevented by cauterizing the wound with a small electric cautery. Owing to faults in the sea-water system during the course of the experiments, mortality among the worms, including our stock of intact specimens, was higher than usual, and infections of worms that had suffered cerebral extirpation or amputation of posterior segments was reduced by adding benzylpenicillin (about 50,000 i.u./litre) to the aquaria from time to time.

RESULTS

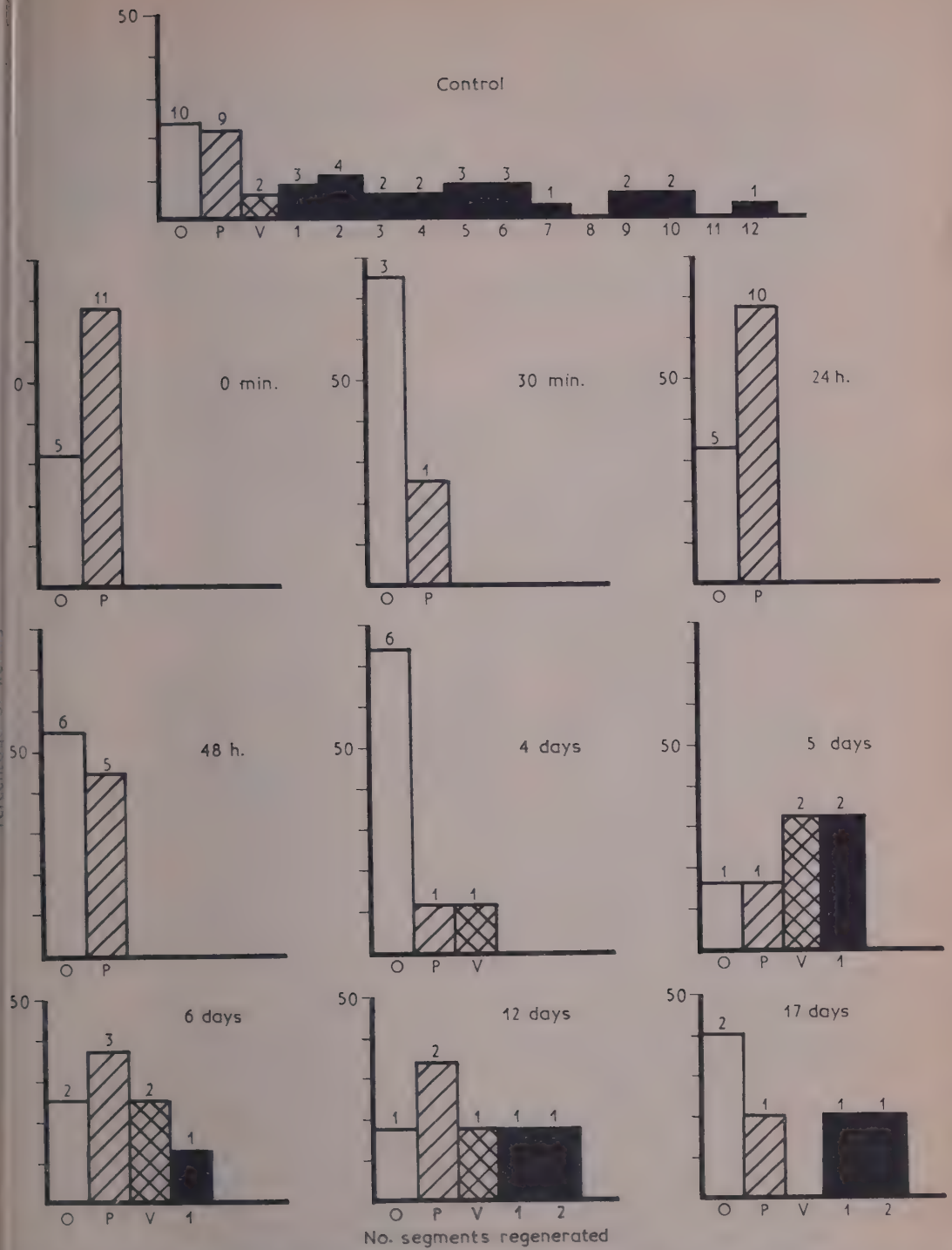
Effect of delayed decerebration upon caudal regeneration

The worms were divided into nine lots (*a–i*) and treated as follows (the numbers in brackets represent the survivors in each group after 30 days).

(*a*) Supra-oesophageal ganglion extirpated before amputation of the posterior segments (16 survivors).

In the remaining groups the supra-oesophageal ganglion was removed after amputation of the posterior segments:

(*b*) 30 minutes after amputation (4);
(*c*) 24 hours after amputation (15);
(*d*) 48 hours after amputation (11);
(*e*) 4 days after amputation (8);



[T]EXT-FIG. 1. Effect upon regeneration of extirpation of the supra-oesophageal ganglion at various times after amputation of posterior segments. *Controls*: ganglion intact throughout. O, no regeneration; P, pygidium regenerated; V, vascularized pygidium regenerated; the succeeding numbers indicate the number of segments proliferated. The number of worms represented by each block is indicated above the block.

(*f*) 5 days after amputation (6); (*h*) 12 days after amputation (6);
(*g*) 6 days after amputation (8); (*i*) 17 days after amputation (5).

In addition, two groups of control worms were set up, corresponding to the two periods when these experiments were carried out. The supra-oesophageal ganglion of the controls remained intact throughout the period of regeneration after amputation of the posterior segments. Of these, 44 survived to the end of the experiment.

All the worms were fixed and examined 30 days from the start of the experiments. The extent of regeneration is shown in Text-fig. 1.

Controls

In earlier experiments (Clark & Bonney, 1960) about 25 per cent of the control worms failed to regenerate even a pygidium, but as the posterior part of the worms became necrotic they were omitted from further consideration. On repeating these experiments we again found that nearly a quarter of the control worms failed to regenerate a pygidium or new segments, but since they remained healthy we are unable to discount them and conclude that some specimens of *N. diversicolor* regenerate extremely slowly or not at all. We are unable to correlate this failure to regenerate with size, sex, or sexual maturity.

Of the remaining worms, 21 regenerated new segments (average 5·0 segments) and 11 a new pygidium, of which 2 were enlarged and vascularized. An average number of 2·6 segments were regenerated by the controls as a whole.

Group (*a*). The conclusion of Clark & Bonney (1960) that extirpation of the ganglion before amputation of the posterior segments prevents segment proliferation, though not the formation of a new pygidium, is confirmed.

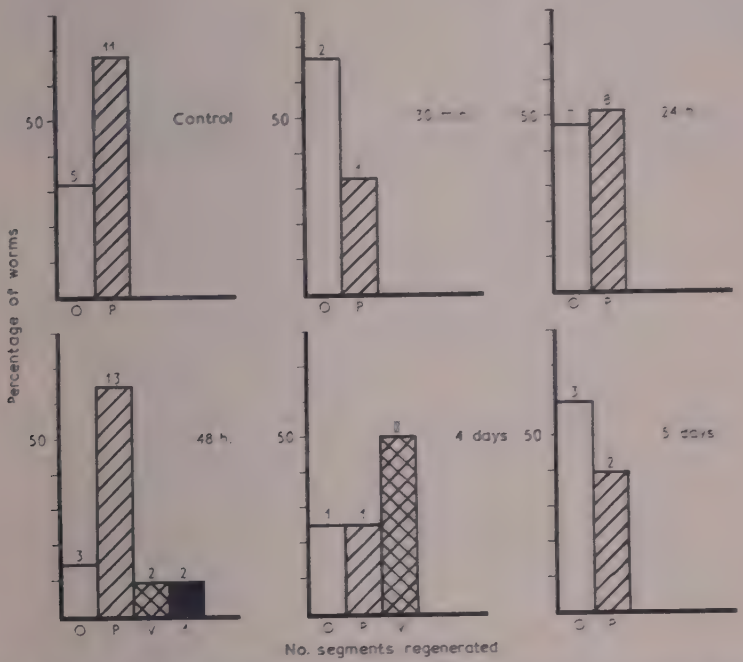
Groups (*b*)–(*d*). Extirpation of the ganglion within 48 hours of amputation of the posterior segments prevents caudal regeneration as completely as decerebration before the amputation of segments. Of the 30 worms in these groups, 16 regenerated a pygidium, but in none was it vascularized.

Groups (*e*)–(*i*). Removal of the ganglion 4 or more days after amputation of the posterior segments does not prevent regeneration, though the number of new segments proliferated is much smaller than that in worms in which the ganglion remains intact. These results may be compared with those obtained previously (Clark & Bonney, 1960). Ganglion extirpation 3 days after amputating posterior segments was then found to result in segment proliferation (a single segment) in 3 of 19 worms; the remainder regenerated at most a pygidium.

Effect of ganglion implantation and injection of ganglion extract during the initial stages of regeneration

The inhibition of regeneration by decerebration either before or shortly after the amputation of a number of posterior segments demonstrated in the previous experiments, and the changes in the neurosecretory activity of the brain of regenerating *N. diversicolor* observed by Clark & Bonney (1960), suggest that

the ganglion is the source of regeneration hormones. We have attempted to confirm this by implanting a whole supra-oesophageal ganglion into the anterior coelom of decerebrate, regenerating worms or by injecting minced ganglion in sterile sea-water into the coelom of the recipient. All the recipients were decerebrated before amputation of the posterior segments and all the donors had been regenerating, with their supra-oesophageal ganglion intact, for the



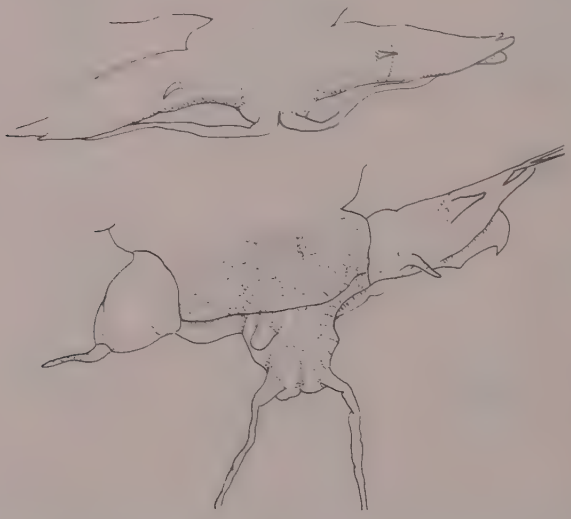
TEXT-FIG. 2. Effect of delayed replacement therapy upon regeneration of posterior segments in decerebrate worms. The time stated is the interval between amputation of the segments and the transplantation or injection of a ganglion from a cerebrate worm that had been regenerating for the same period. Controls: decerebrate worms. The number of worms represented by each block is indicated above the block.

same length of time as the recipient, when the ganglion was removed for implantation. The majority of worms into which foreign ganglia were implanted died, probably because they had to undergo a double operation, once when decerebrated and again when the wound was reopened and the ganglion implanted. We have therefore relied chiefly upon injection of minced ganglion which, although it involves a second narcotization of the recipient, is not a lethal operation.

Implantations were made 30 minutes (3 survivors) and 48 hours (6 survivors) after amputation of posterior segments. Injections were made after intervals of 24 hours (15 survivors), 48 hours (14 survivors), 4 days (4 survivors), and

5 days (5 survivors) following amputation. All the recipients were examined 30 days after the loss of the posterior segments and a number of worms were maintained for an additional 20 days. The amount of regeneration at the end of 30 days in each of these groups of worms is shown in Text-fig. 2. There was no detectable difference between the effects of the two methods of replacing ganglionic material and the results of both series of experiments have been amalgamated.

Implantation or injection of ganglionic material during the first 24 hours of regeneration does not induce the formation of new segments. Nothing more than a pygidium was formed in these worms, and not even that in a third of them. This is comparable to the extent of regeneration in worms decerebrated before or up to 48 hours after amputation of the posterior segments (cf. Text-fig. 1).



TEXT-FIG. 3. *Upper*: dorsal view of a regenerated pygidium and anal cirri of a decerebrate worm. *Lower*: ventral view of a regenerated, vascularized pygidium with anal cirri and a unilateral parapodial rudiment in a decerebrate worm, injected with ganglion extract 48 hours after amputation of the posterior segments.

Replacement of ganglionic material 2–4 days after amputation of the posterior segments was followed within 30 days by the formation of a new pygidium in the great majority of worms, by the formation of a large vascularized pygidium in 4 specimens, and by the appearance of a pair of parapodial rudiments in 2 others (Text-fig. 3). These worms were kept for another 20 days before fixation, but no further regeneration took place in that time.

Replacement therapy on the 5th day appears to be unsuccessful, but since only a small number of worms in this group survived to the 30th day it is probably safer to ignore this result.

DISCUSSION

It is clear from the first series of experiments that the supra-oesophageal ganglion exerts a decisive role in initiating regeneration, and that it must be intact for at least 48 hours after injury for any new segments to be regenerated. However, although new segments may be formed if the ganglion remains intact for 3 days or more, removal of the ganglion at any time during the first 2 weeks after the loss of posterior segments retards regeneration, indicating that the ganglion continues to exert some influence upon regeneration during this period.

That the influence of the ganglion is hormonal is confirmed by the second series of experiments. Implantation of the ganglion, or the injection of ganglion extract, from worms that have themselves been regenerating for 3 or 4 days into decerebrate worms that have lacked the posterior segments for a similar period, sometimes results in the regeneration of a vascularized pygidium or a single pair of parapodial rudiments in the latter. The replacement of the cerebral regeneration hormones is obviously very incomplete, since no more than one segment was regenerated although the worms were kept under observation for a total of 50 days. However, implantation or injection of a single ganglion results in an important advance in the regenerative processes over those seen in decerebrate worms, in which a vascularized pygidium is never formed and segment proliferation has never been observed. The initial stages of regeneration in *N. diversicolor*, as in other annelids, are marked by the migration of free coelomic cells to the wound (Nusbaum, 1908; Dehorne, 1950). In the absence of such a migration a new pygidium with anal cirri may be formed by the reorganization of tissues bordering the wound, but in *Nereis* mesodermal derivatives, including blood-vessels, are not formed, and segment proliferation does not follow upon the formation of a new pygidium (Stéphan-Dubois, 1956). Stéphan-Dubois also demonstrated that the regeneration of an enlarged, vascularized pygidium is a characteristic intermediate stage between normal regeneration with segment proliferation and local reorganization of the epidermal tissues to form only a pygidium. Our results are comparable. Replacement of ganglionic material during the first 2 days after injury is followed by a degree of regeneration characteristic of that when coelomic cells have not migrated to the wound. Replacement on the 3rd or 4th days is followed by regeneration characteristic of a limited migration of the coelomic cells.

We can also draw certain conclusions about the sequence of events following the loss of the posterior segments. Injury is followed by a response on the neurosecretory cells of the supra-oesophageal ganglion within 6–12 hours (Clark & Bonney, 1960). However, regeneration hormones are either not elaborated or not released in sufficient quantity during the first 48 hours to initiate regeneration, because extirpation of the ganglion during this period prevents subsequent regeneration. The fact that replacement of ganglionic material, whether by

implantation of a whole ganglion or by injection of minced ganglion, is incapable of inducing regeneration, suggests that the hormones have not yet been elaborated in quantity, and that the ganglion must remain intact for this to happen. The period around 48 hours after injury appears to be a critical one when the hormones are present in the ganglion (replacement is partially successful in inducing regeneration) but have not yet been released (extirpation of the ganglion at this time still inhibits regeneration). By the 3rd (Clark & Bonney, 1960) or 4th days (present results) the hormones are evidently circulating in the blood or body fluid of the animal, because brain extirpation no longer totally inhibits regeneration. The ganglion is still a source of the hormone and replacement of ganglionic material at this stage induces regeneration in decerebrate worms. Whether by the 5th day the ganglion ceases to secrete the regeneration hormones remains to be investigated. The results of the brain-extirpation experiments suggest that it continues to promote regeneration at least until the 17th day and probably longer, and the contrary evidence of the failure of replacement therapy on the 5th day is probably too slender to be considered at present.

SUMMARY

1. Extirpation of the supra-oesophageal ganglion of *Nereis diversicolor* before or up to 48 hours after amputation of a number of posterior segments inhibits caudal regeneration. Extirpation of the ganglion 3 or more days after amputation of segments retards but does not inhibit regeneration.

2. Injection or implantation of supra-oesophageal ganglia of regenerating worms into decerebrate hosts 48 hours or more after amputating the posterior segments results in segment proliferation in some of the recipients. Replacement of ganglionic material before 48 hours has no effect.

3. The types of regeneration observed following replacement therapy on the 3rd or 4th days is characteristic of that when there has been a limited migration of coelomic cells to the wound.

4. 'Regeneration' hormones appear to be present in the ganglion 48 hours after the loss of posterior segments, but they do not circulate in the body in effective quantity until 24 hours later.

RÉSUMÉ

L'Effet de l'extirpation et du remplacement différés du cerveau sur la régénération caudale de Nereis diversicolor

1. Si l'on extirpe le ganglion supra-œsophagien de *Nereis diversicolor* avant l'amputation d'un certain nombre de segments postérieurs, ou jusqu'à 48 heures après l'amputation de ces mêmes segments, la régénération est inhibée.

2. L'extirpation du ganglion plus de 48 heures après l'amputation des segments retarde mais n'empêche pas la régénération.

3. Le remplacement, par implantation ou injection, du matériel ganglionnaire des vers décérébrés, pendant les 24 premières heures après l'amputation n'est pas suivi de régénération. La même opération, pratiquée 48 heures ou plus après l'amputation, donne lieu à quelque régénération, en particulier à la prolifération de segments.

4. On peut conclure de ces expériences que des hormones de régénération sont élaborées dans le ganglion supra-œsophagien pendant les 2 ou 3 premiers jours après la perte des segments postérieurs. A 48 heures, elles sont présentes dans le ganglion, mais elles ne circulent pas en assez grande quantité dans le sang ou dans le liquide cœlomique pour provoquer la régénération. Vers 72 heures, la régénération est amorcée, mais le ganglion continue à exercer son influence sur la régénération pour une période d'au moins 14 jours.

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Placental Grafts in Rats

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WITH TWO PLATES

INTRODUCTION

We have previously reported that certain parts of the rat placenta are capable of growth and differentiation when transferred from the uterus to the mother's omentum (Payne & Payne, 1960). Yolk-sac membrane, transplanted on the 15th day of pregnancy, produced grafts containing a variety of tissues which included epidermoid cysts, mucus-secreting epithelium, muscle, bone, and cartilage. The purpose of this paper is to describe the work in detail and to report the results of further experiments.

METHODS

Stock female rats (Albino Wistar strain) were used throughout except in 2 experiments where male rats of the same strain or stock albino mice were employed. The rats came from a colony at Compton which was not inbred although the population had been 'closed' for many years and bulk breeding had been practised. All animals were mature when incorporated in the experiments, rats weighing 200–250 g. and mice 20–25 g.

Mating of animals

Males were caged with the female rats and vaginal smears taken daily. When spermatozoa were found this was taken to indicate successful mating and the date of conception was recorded. Mated females were then transferred to cages labelled with this date so that they could be incorporated in experiments when the appropriate stage of pregnancy had been reached.

Procedure for grafting

Each experiment involved the removal of the uterus from a pregnant rat, the dissection of the placentae and the grafting of certain portions into the mother or another host. Surgery was carried out under general anaesthesia with strict aseptic precautions.

Anaesthesia. Various anaesthetics were used in order to find one which would perhaps be safer and more acceptable to the rat than the conventional open

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ether method. In most experiments anaesthesia was induced by 'Avertin' (tribromoethanol in amylene hydrate, Bayer Ltd.) or 'Cyclonal Sodium' (hexobarbitone sodium BPC, May & Baker Ltd.) given intraperitoneally and supplemented where necessary by the volatile anaesthetics, 'Vam' (mixture of vinyl and ethyl ether, May & Baker Ltd.) or 'Trilene' (trichloro-ethylene BP, I.C.I. Ltd.). Experiments indicated that the choice of anaesthetic had no demonstrable effect on the results of grafting.

Hysterectomy. The gravid uterus was removed by means of a midline laparotomy, haemorrhage being prevented by ligation of the uterine blood-vessels. The uterus was then transferred for dissection to a sterile Petri dish. Meanwhile, the mother's abdominal wound was left open for a few minutes until placental grafts could be prepared for transplantation to the omentum. In certain experiments indicated in the text the ovaries were also removed.

Dissection of the uterus. The uterine wall was carefully opened with scissors so as to expose the conceptuses undamaged. The disk-shaped chorio-allantois could then be seen attached mesometrially, whilst beneath hung the visceral yolk-sac wall containing fluid and the foetus. The yolk sac was then cut near its point of attachment to the disk and opened so that the foetus and contained fluid flowed out. The attachments of the foetus to the membranes were now severed and the foetus removed, leaving the visceral yolk-sac wall, amnion, and possibly a little umbilical cord. Further dissection to remove the amnion was carried out in Ringer fluid over a dark background so that the yolk sac could be identified as a thick membrane with blood-vessels in its wall as compared with the amnion, which was thin, flimsy, and avascular. Tissue deemed to be pure yolk sac was used for grafting, but histological sections of such material frequently revealed the presence of small pieces of amnion and even umbilical cord. Contamination with tissue from the foetus was not observed. Plate 1, fig. B illustrates the histological appearance of the yolk sac and fragments of attached amnion.

Most grafts were taken from the uteri on the 14th or 15th day of pregnancy but, where indicated in the text, material was also taken on the 11th or 18th day. Accurate dissection was not possible on the 11th day, when all preparations were found to be mixtures of various placental and foetal tissues. On the 18th day dissection was relatively easy, for by then the various membranes were well formed.

Grafting technique. One-half of a yolk sac as prepared above is sufficient for a successful 'take', but as ample material was available one yolk-sac preparation was used for each host. If the host was also the donor mother-rat a fold of omentum was gently lifted out through the same incision as that used for the hysterectomy. For other hosts a small midline incision was made specially for the grafting procedure. The fold of omentum was spread fanwise on a sterile cloth and a yolk-sac preparation laid upon it. The omentum was then wrapped over so as to completely enclose the transplant and the whole structure returned

to the abdominal cavity. Grafts remained where placed and no further anchoring was necessary. All abdominal wounds were closed by two layers of sutures.

Modifications of this basic technique were employed in certain experiments mentioned later. These included transplanting rat yolk-sac preparations to the omentum of mice, and adult skin, foetal skin, or amnion to the omentum of rats.

Necropsy. Animals were killed at intervals of 1, 2, and 3 months after operation. The gross appearance of the graft was recorded before fixation in 10 per cent. formal saline. Histological sections were prepared and stained with Ehrlich's acid haematoxylin and eosin or with special stains to demonstrate particular features.

RESULTS

The macroscopic and microscopic appearance of grafts varied from rat to rat, but a number of well-defined patterns of growth commonly occurred. These will now be described.

Typical macroscopic appearance of grafts of yolk-sac preparations taken on the 14th–15th day of pregnancy

At 1–3 months after transplantation the grafts varied in size and appearance but usually took the form of cysts with nodular bulges or convolutions projecting from the surface. The largest attained a diameter of 5 cm. (Plate 1, fig. A) but $\frac{1}{2}$–1 cm. was a more usual size. The cysts were filled with mucus which was either clear or turbid and yellow. Some grafts were not cystic but consisted of a series of small granular masses which on histological examination were usually found to be epidermoids. In cases where the graft had not grown there was usually no visible remnant of the original transplant or a tiny nodule of granulation tissue might be observed. No differences were observed between grafts in the mother and in other stock rats.

Typical microscopic appearance of grafts of yolk-sac preparations taken on the 14th–15th day of pregnancy

Although a variety of tissues developed in the grafts there were four main structural patterns: (*a*) the cysts mentioned above, which usually resembled intestinal wall, (*b*) skin, and skin derivatives, (*c*) skeletal tissues such as cartilage or bone, and (*d*) various kinds of connective tissue. These four types of growth could occur separately, or in any combination in the same graft.

Cysts resembling intestinal wall

These were the commonest structures found. They contain mucin, which stains red with mucicarmine and PAS stains, together with a few clumps of degenerating columnar cells which have apparently broken away from the cyst wall. The lining epithelium, in which there are often many dividing cells, is usually of the tall mucus-secreting type with many goblet cells (Plate 1, fig. C).

It is rarely smooth, but thrown into folds closely resembling intestinal villi (Plate 1, fig. D), each covered by columnar epithelium around a core of connective tissue containing histologically normal and functional blood-vessels. At the base of the villi are cells which differ from the rest of the epithelium in that their cytoplasm contains masses of coarse eosinophilic granules (Plate 1, fig. D). They closely resemble the cells of Paneth which occur in the small intestine.

The connective tissue of the cyst wall may contain cells such as lymphocytes, polymorphs, and macrophages which are usually associated with inflammatory reaction. This might well be due to a homograft reaction by the host against graft antigens. It may also be the result of excessive secretion of mucin, which ruptures the cyst wall and escapes into the surrounding tissues. In addition, secondary bacterial infection may have supervened in old, very distended cysts, so that the contents become converted to muco-pus and the lining epithelium severely ulcerated. In both cases the epithelium actively attempts to heal the eroded areas.

Cysts are nearly always surrounded by thick bands of smooth muscle in which the fibres are orientated in transverse and longitudinal layers. In some well-developed cysts there may even be a rudiment of muscularis mucosa, but this is rare. The resemblance to intestinal structure is completed by clusters of neurones between the muscle layers reminiscent of the myenteric plexus of Auerbach (Plate 1, fig. E).

Whilst the above description is that of the structure of a typical cyst, several variations do occur. Some cysts are simple and composed only of mucus-secreting epithelium and thickened strands of hyaline fibrous tissue (Plate 1, figs. F, G). It is convenient to mention here that this type of connective tissue is a common component of yolk-sac grafts; it resembles, and appears to be derived from, Reichert's membrane, which is a normal structure in the yolk sac of rat placentae.

Skin and skin derivatives

Simple round epidermoids are the next most common structure found in the grafts. They contain a centre of keratin surrounded by all the structural layers commonly present in mature skin (Plate 1, fig. G; Plate 2, figs. H, I); from the periphery inwards can be traced the strata germinativum, granulosum, lucidum, and corneum. Mitoses are common in the basal layers. In rare cases structures akin to skin derivatives, such as hair follicles with hair and sebaceous glands, are produced (Plate 2, fig. J).

Skeletal structures

These are rare as compared with mucus-secreting cysts and epidermoids (see Table). In their simplest form they occur as small round nodules of cartilage (Plate 2, fig. K). Larger pieces of cartilage undergo central degeneration or

endochondrial ossification (Plate 2, fig. L). In different grafts stages in bone formation may be traced from preliminary ossification to the development of structures closely resembling long bones with cartilaginous extremities and ossified shafts. Haemopoietic cells are present in the medulla of one such bone.

TABLE

All grafts transplanted to omentum unless otherwise stated

Factors investigated	*Day of pregnancy when yolk sac taken*	*Host*	*No. takes*	*No. failures*	*Graft constituents*
Host	14th day	Mothers	4	4	4M, 3E
	,,	Stock females	3	5	2M, 1C
	,,	Ovariectomized stock	3	5	3M
	,,	Males	6	7	4M, 4E
Age of yolk sac	11th day	Stock females	2	23	2M, 1C, 1B
	14th day	,,	15	10	15M, 8E, 1C, 1B
	18th day	,,	3	21	3E
Age of graft	14th day	Stock females killed after 1 month	3	5	2M, 1C
	,,	Stock females killed after 2 months	8	1	8M, 6E, 1B
	,,	Stock females killed after 3 months	4	4	5M, 2E
Transplantation site	,,	Stock females subcuta grafts to 6 sites neons	8	13	7M, 1E, 1C, 1B

M, mucus-secreting cyst; E, epidermoid cyst; C, cartilage; B, bone.

Connective-tissue stroma of the grafts

The various components of the graft are usually bound together into a discrete mass by fibrous tissue. Sometimes this is mature and collagenous, staining red in Van Gieson preparations, but at other times there is relatively little collagen and mesenchyme cells predominate. Thick strands of hyaline connective tissue (Plate 1, figs. F, G) resembling Reichert's membrane occur in most grafts even when no other placental structures have grown.

Experiments to determine the factors concerned in successful placenta-grafting

The importance of the sex and hormone status of the host

Our initial report (Payne & Payne, 1960) described the grafting of yolk-sac preparations to the omentum of the donor mother. In later experiments it was shown that grafts grow equally well in other hosts, i.e. stock female rats, intact males, and ovariectomized females. The results of these experiments are summarized in the Table.

The stage of pregnancy at which the yolk sac is removed

Yolk sacs taken on the 14th or 15th day of pregnancy will grow successfully when transplanted. The Table records the corresponding results for yolk sacs

removed from the rats on the 11th, 14th, and 18th day of pregnancy. Very few 'takes' occurred with the 11th- and 18th-day material, and these were all small and poorly differentiated. One of the mucus-secreting cysts in the 11-day group was curious in that its lining consisted of mucus-secreting cells interspersed with areas of stratified squamous epithelium (Plate 2, fig. M).

The site for transplantation

In most of the experiments yolk-sac preparations were transplanted into the omentum. This involved a short but tedious surgical operation which might have been avoided had subcutaneous implantation proved successful. However, the Table records the relatively inferior results obtained when 14-day yolk sacs were implanted into 6 subcutaneous sites in each of 21 stock female rats.

Time allowed for development of graft

Groups of rats which received 14-day yolk-sac preparations were killed at 1, 2, and 3 months after operation. The Table records that the number of successful grafts did not decrease as time progressed to 3 months. Though some of the grafts examined at 1 month showed inflammation, this was not a prominent feature of those taken at 2 and 3 months, when the various structures were better differentiated.

Grafts of rat yolk-sac preparations to mice

Rat yolk-sac preparations were transplanted into the omentum of each of 47 stock mice. When the animals were killed 1 to 2 months later there was no evidence of any transplant growth, whereas yolk-sac preparations taken from the same donor rats and grafted to the omentum of rats grew successfully.

The homogeneity of the rat stock

In this experiment portions of shaved abdominal skin were removed from rats and divided into two parts. One part was transplanted to the omentum of the donor rat as an autograft and the other to the omentum of another stock rat to form a homograft. Ten pairs of such animals were used in the experiment. When the rats were killed 1 month later all the autografts were healthy but only 1 homograft had grown; 5 homografts were represented by remnants of degenerating skin, whilst the other grafts were entirely replaced by granulation tissue resulting from chronic inflammatory reaction.

Grafts of foetal skin

As adult homografts were rarely successful in our stock rats the lack of homograft reaction to placental tissue must be due either to a particular propensity of placental tissue for grafting or to a property of foetal tissue in general. In an attempt to clarify this point portions of foetal rat limbs were taken on the 14th day of pregnancy and grafted into the omentum of stock rats which were kept for 2 months and then autopsied. Of 13 such grafts all grew and produced skin, skin derivatives, and bone, but evidence of skin degeneration was present in four.

Attempts to graft chorio-allantoic placenta

In spite of repeated attempts, we have been unable to produce a successful graft of chorio-allantois. Cells of the developing chorio-allantoic placenta were included in yolk-sac preparations from 11-day pregnant rats (see above) but the trophoblast cells ceased to divide and none survived for more than a few days. Slices of chorio-allantoic disk taken at 14 days' pregnancy and grafted to the omentum failed to grow. Implantation of similar material into the spleen was also without success. Finally, in each of three pregnant animals undergoing partial hysterectomy one placenta proximal to an ovary was left *in situ* and covered with omentum. By this procedure the placenta had every chance of survival as its blood-supply was intact, but at necropsy 4 weeks later only a necrotic remnant was found.

Grafts of amnion

As a certain amount of amnion was known to be included in yolk-sac preparations, it was decided to determine how it would develop if grafted alone in the same way as the yolk-sac preparation. Thirteen stock female rats received pieces of this membrane which had been carefully dissected from the placentae of mothers on the 15th day of pregnancy. Two months later only one graft had grown and this had produced a small simple epidermoid cyst.

DISCUSSION

There are very few references in the literature to the successful grafting of placental membranes. Peer (1959) quotes the experimental work of Douglas *et al.* (1954), which suggests that foetal membrane grafts are tolerated for 2 to 3 times the average survival time of skin homografts. Foetal membrane transplants in mice were apparently viable and capable of undergoing cellular division and epithelization when observed by the transparent tissue chamber technique. Willis (1958), in a review of the literature, described his own and other's experiments on the transplantation of embryonic tissues but gave no reference to any work which had resulted in the successful grafting of placental membranes. He states that a wide range of foetal tissues have been transplanted and that homografts of foetal origin survive and differentiate whereas similar adult tissues are rejected.

Our previous communication (Payne & Payne, 1960) recorded that yolk-sac membranes transferred from the uterus to the omentum of the mother rat grew and differentiated into a variety of tissues. The present paper describes further experiments which confirm and extend our knowledge of this original finding.

The first subject for discussion is the origin of the various types of tissue which we have found in the grafts. These have included mucus-secreting cysts which closely resemble intestine, epidermoid cysts, and skin derivatives, together with skeletal elements such as bone and cartilage. The origin of tissues resembling intestine is perhaps the easiest to explain, in that the yolk sac is composed of

endoderm and mesoderm cells embryologically related to the gut. It seems that the yolk sac at the 14th–15th day of pregnancy retains its potentiality for differentiation into intestine, but that by the 18th day of pregnancy it loses this capacity, as grafts taken at this stage rarely grow. Alternatively, it may be that by the 18th day the placenta is capable of inducing a homograft reaction which prevents its growth.

The growth of skin is more difficult to explain. As there is no ectoderm in the yolk sac the possible sources are from tissue other than yolk sac included in the graft, or metaplasia of yolk-sac endoderm. On the basis of present evidence neither source can be said to be entirely confirmed or excluded. The amnion has cells of ectodermal origin, and it has been shown to contaminate yolk-sac preparations used in these experiments; when isolated and grafted without yolk sac it did produce an epidermoid cyst in one case. However, metaplasia of yolk-sac endoderm cannot be ruled out.

The origin of skeletal structures in the grafts is particularly difficult to explain. There are two possibilities: either that cartilage or bone-producing elements are present in the original transplant, or that these tissues are produced from host cells. It seems unlikely that they could have arisen from the transplanted cells, for the mesoderm associated with the placental membranes has little connexion with the skeletal mesoderm of the embryo. In support of the alternative hypothesis, induction of bone in the omentum was described by Huggins (1931), who transplanted pieces of bladder wall to the omentum and found that membrane bone developed in the neighbouring tissues. Later, Huggins & Sammet (1933) showed that gall-bladder epithelium also had osteogenic potentialities. It is just possible that yolk-sac grafts might induce bone formation in the same way, but it must be stressed that the bone which developed in Huggins's experiments was membrane bone, whereas cartilage and endochondral ossification occurred in the present experiments. Even though yolk-sac mesoderm is not skeletal, in an unusual environment it might produce bone and cartilage like the omentum mesoderm in Huggins's experiments. The discrete nature and morphology of the skeletal derivatives in our yolk-sac grafts suggest origin from the graft itself, but this may be a misleading impression. Until further evidence is available the origin of bone and cartilage in the grafts remains uncertain.

An aspect of the work which must be stressed is the importance of taking the placenta at about the 14th or 15th day of gestation. Very few successful results were obtained with tissue taken on the 11th and 18th day. It may thus be assumed that the period of potentiality for growth and differentiation of tissue from the yolk sac is circumscribed; it is not attained by the 11th day and by the 18th day the yolk sac is apparently incapable of growth in the new environment.

Our previous work (Payne & Payne, 1951) reported the successful grafting of yolk-sac preparations into the omentum of the donor mother. The present paper presents evidence that similar grafts grow equally well in other stock females, whether the ovaries are present or absent, and also in stock male rats. It

therefore seems unlikely that the sex-hormone environment of the host tissue has any effect on the development of yolk-sac grafts.

Placental grafts are homografts even if they are implanted into the donor mother and their successful growth raised problems of tissue tolerance. It was therefore necessary to assess how far our stock rats were capable of accepting tissues transplanted from one to another. Our experiments indicated that homografts of adult skin, when transplanted into the omentum, were rejected or underwent homograft reaction, whereas foetal skin was comparatively well tolerated. These results are similar to those of Toolan (1957), who found that foetal tissue could be successfully transplanted to homologous adults. Thus it is likely that the tolerance of adult rats to placental tissues is part of a wider phenomenon, that of tolerance of adult animals to foetal tissues in general. The tolerance does not extend, however, to heterografts, for rat yolk-sac preparations transplanted to mice failed to grow. Further experiments are in progress to investigate the problem of tolerance, using inbred strains of rats.

SUMMARY

Yolk sacs from rat placental membranes taken on the 14th or 15th day of pregnancy grew and differentiated in a remarkable manner when transplanted into the mother's omentum. A variety of tissues developed within them, including mucus-secreting cysts, epidermoid cysts, cartilage, and bone. The mucus-secreting cysts were surrounded by smooth muscle and closely resembled intestine; they probably arose from the endoderm and mesoderm of the yolk sac. The epidermoids could have originated either from amnion remnants included accidentally in the transplant, or from metaplasia of the yolk-sac endoderm. It is obscure whether the cartilage and bone developed from host or graft. Transplants were rarely successful when removed from rats at the 11th or 18th day of gestation.

Similar results were obtained when the hosts were other stock rats, either normal or ovariectomized females or males.

The grafts were well tolerated by the hosts and homograft reactions did not occur. Members of the rat stock used would not tolerate adult skin homografts in the omentum, but transplants of foetal skin and bone grew and differentiated well. It is concluded that the tolerance of yolk-sac membranes is not a characteristic peculiar to placental tissue, but is part of a wider phenomenon of tolerance to foetal tissues in general.

RÉSUMÉ

Greffes placentaires chez le Rat

La croissance et la différenciation de sacs vitellins de rat, prélevés dans les membranes placentaires le 14e ou le 15e jour de la gestation, se sont remarquablement effectuées après transplantation de ces sacs dans l'épiploon de la mère.

Divers tissus se sont développés dans ces transplants, comprenant des kystes sécréteurs de mucus, des kystes épidermoïdes, du cartilage et de l'os. Les kystes muqueux étaient entourés de muscles lisses et ressemblaient étroitement à de l'intestin; ils provenaient vraisemblablement de l'endoderme et du mésoderme du sac vitellin. Les kystes épidermoïdes pourraient avoir pris naissance à partir de restes d'amnios inclus accidentellement dans le transplant, ou d'une métaplasie de l'endoderme du sac vitellin. On ne sait si le cartilage et l'os se sont formés à partir de l'hôte ou à partir du greffon.

Les transplantations ont été rarement réussies quand les prélèvements ont été faits le 11e ou le 18e jour de la gestation.

Des résultats semblables ont été obtenus en prenant pour hôtes des rats d'autres souches, femelles normales ou ovariectomisées et mâles.

Les hôtes ont bien toléré les greffes et il n'y a pas eu de réaction d'homogreffe. Les individus de la souche de rats utilisée ne toléreraient pas d'homogreffes de peau adulte dans l'épiploon, mais des transplants de peau et d'os fœtaux s'y sont accrus et se sont bien différenciés. On en conclut que la tolérance à l'égard des membranes du sac vitellin n'est pas une caractéristique particulière au tissu placentaire, mais fait partie d'un phénomène plus étendu de tolérance envers les tissus fœtaux en général.

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EXPLANATION OF PLATES

Plate 1

Fig. A. Large mucus-secreting cyst removed from the omentum 3 months after transplantation. The wall has been cut so as to allow the mucoid contents to escape. ×4·9.

Fig. B. Yolk sac and amnion membranes taken on the 15th day of pregnancy. The yolk sac is thick and contains blood-vessels, whereas the amnion is thin. Haematoxylin and eosin. ×300.

FIG. C. Mucus-secreting cyst 2 months after transplantation. Goblet cells containing mucin are present in the villi of the lining epithelium. Periodic acid Schiff stain. ×300.

FIG. D. Wall of mucus-secreting cyst. At the base of the villi are cells closely resembling cells of Paneth. Smooth muscle is visible towards the edge of the photograph. Haematoxylin and eosin. ×300.

FIG. E. Wall of mucus-secreting cyst. A group of neurones which resemble part of the myenteric plexus of Auerbach can be seen. Haematoxylin and eosin. ×300.

FIG. F. A simple mucus-secreting cyst surrounded by thickened strands of hyaline fibrous tissue. Haematoxylin and eosin. ×300.

FIG. G. A simple mucus-secreting cyst and an epidermoid in the same graft separated by thickened strands of hyaline fibrous tissue. Haematoxylin and eosin. ×300.

PLATE 2

FIG. H. A simple epidermoid cyst. The cell layers normally associated with mature skin are present. Haematoxylin and eosin. ×300.

FIG. I. An epidermoid cyst the wall of which appears to be developing a hair follicle or gland. A mitotic figure can be seen. Haematoxylin and eosin. ×300.

FIG. J. An epidermoid cyst in which hair and sebaceous glands have been formed. Haematoxylin and eosin. ×300.

FIG. K. Round nodules of cartilage present in a graft. Haematoxylin and eosin. ×300.

FIG. L. A graft containing a piece of cartilage which is undergoing endochondrial ossification. Haematoxylin and eosin. ×300.

FIG. M. The wall of a cyst which is lined by mucus-secreting cells and also by stratified squamous epithelium. Haematoxylin and eosin. ×300.

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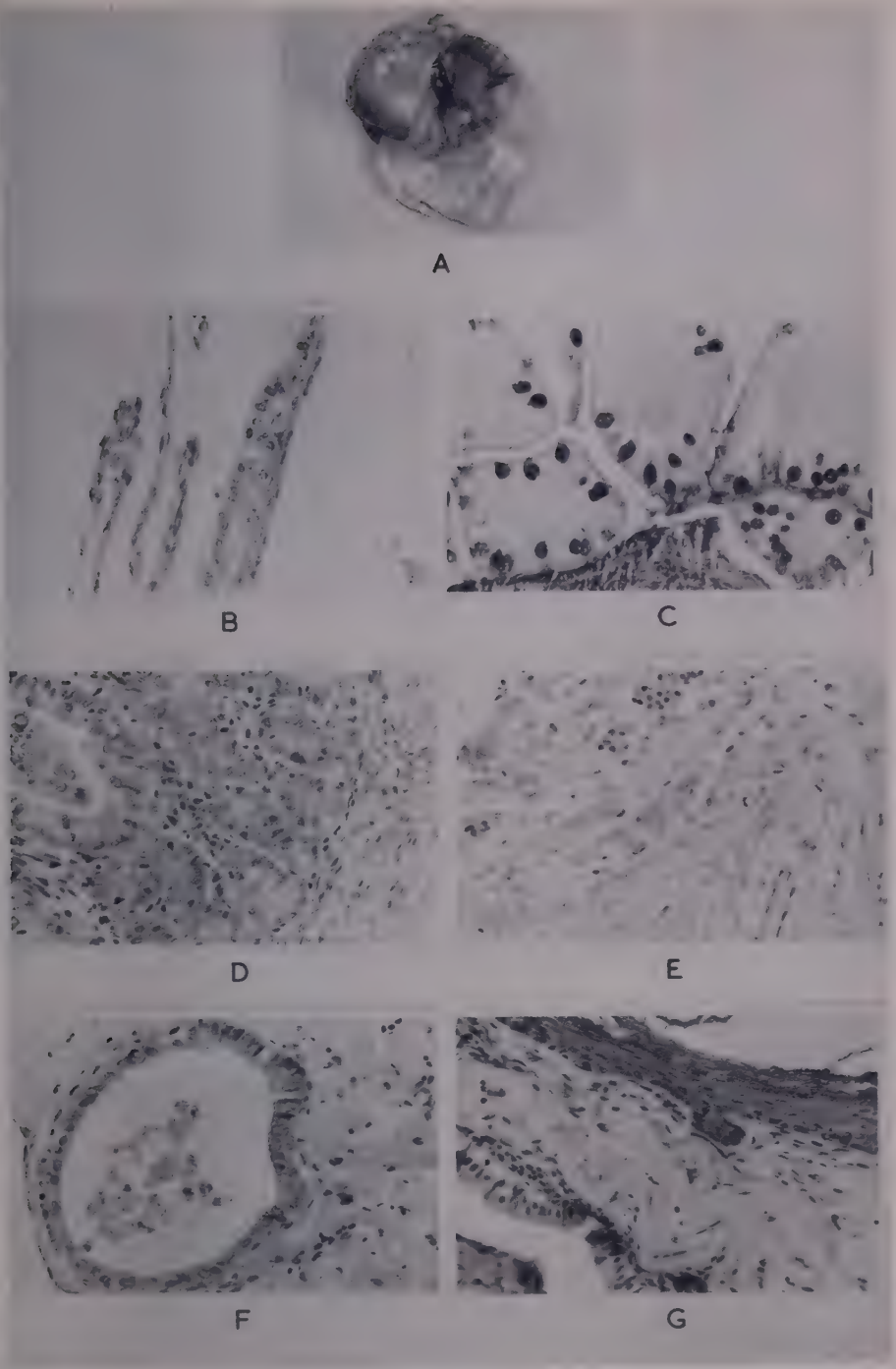
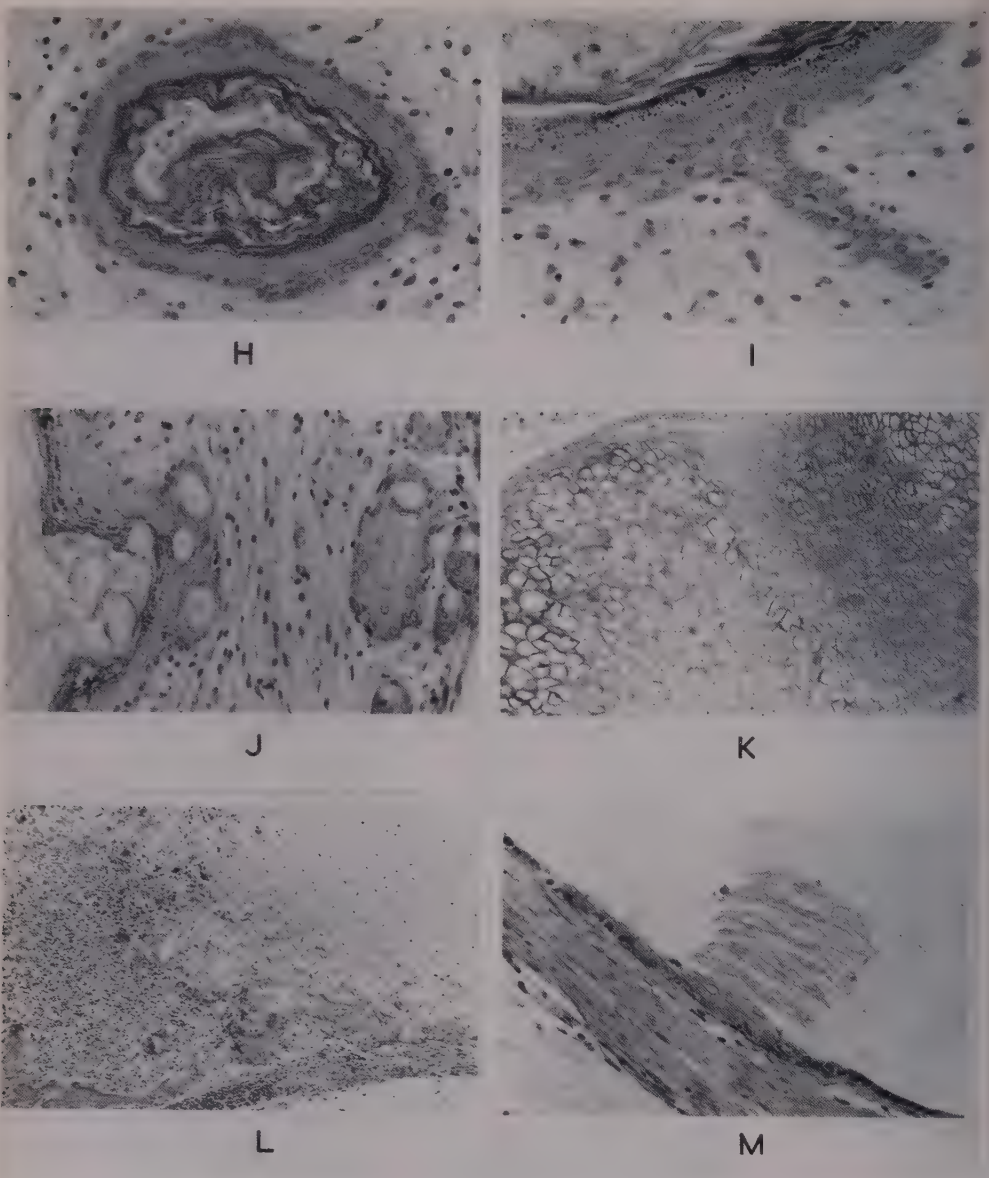


J. M. PAYNE *and* S. PAYNE

Plate 1



J. M. PAYNE *and* S. PAYNE

Plate 2

The Transplantation of Individual Rat and Guinea-pig Whisker Papillae

by J. COHEN[1]

From the Department of Zoology and Comparative Physiology, University of Birmingham

WITH TWO PLATES

INTRODUCTION

PREVIOUS work on the pigmentation and transplantation of hair and skin has been performed mainly on gross skin areas (e.g. Billingham & Medawar, 1950; Ebling & Johnson, 1959; Rawles, 1955) and several studies have been concerned with the histological appearances of various stages in hair development, sometimes correlated with the effects of chemical or physical agents (e.g. Chase, 1955; Chase, Rauch & Smith, 1951). So far there have been no reports of successful transplantation of individual hair papillae, although several authors have considered this to be a possibility (e.g. Billingham, 1958). Lillie & Wang (1941, 1944) showed that a feather papilla may produce generations of feathers after transplantation to another follicle, and that the feathers produced from a transplanted papilla containing both dermal and epidermal components ('whole papilla') are of donor tract structure and colour; feathers produced from local ectoderm under the influence of a transplanted dermal papilla are of host tract structure and colour. It was considered that such an approach to the study of the epigenetic relationships involved in the growth of hair might throw some light on such problems as the precise functions of the dermal papilla, the mechanism by which hair follicles produce different kinds of hair, and the functional morphology of the hair-cycle.

Several attempts have been made to determine the relationship of the dermal papilla to hair growth. Crounse & Stengle (1959) have transplanted human 'hair roots', in Millipore chambers, to the peritoneal cavities of mice, with and without the dermal papillae. Sixteen of 28 transplants including dermal papillae survived, but all 20 deprived of their dermal papillae degenerated. It was concluded that the dermal papilla is necessary for maintenance of the organization of the 'hair root'. As a result of X-irradiation experiments Geary (1952) concluded that it is the destruction or disorganization of the dermal papilla which results in the permanent hair loss found after intense irradiation, while temporary

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epilation results from an action on the matrix (ectodermal part of the papilla). Van Scott & Ekel (1958) have correlated the number of mitoses in the matrix and its volume with the number of cells in the dermal papilla and with its volume, in normal human scalp-hair, in early male baldness, in alopecia areata, and after therapy of various kinds. They found correlations between the measurements of dermal papillae and those of the matrix, and consider these evidence of an epigenetic effect. Chase (1954) considered the dermal papilla of the hair to be an inductive agent comparable with that of the feather, and believed that the dermal papilla maintains its integrity throughout the growth-cycle.

Because of the large size of their papillae it was decided to confine experiments to the whiskers until such time as the technique had been developed sufficiently to deal with the tiny papillae of the pelage. The whisker appears to resemble a guard hair of the pelage very closely except, of course, for its size, its large perifollicular blood sinus, and its considerable innervation (Melaragno & Montagna, 1953; Vincent, 1913). An additional advantage accruing from the use of whiskers is the easy recognition of donor type hair among the hairs of the implantation site.

EXPERIMENTAL TECHNIQUE

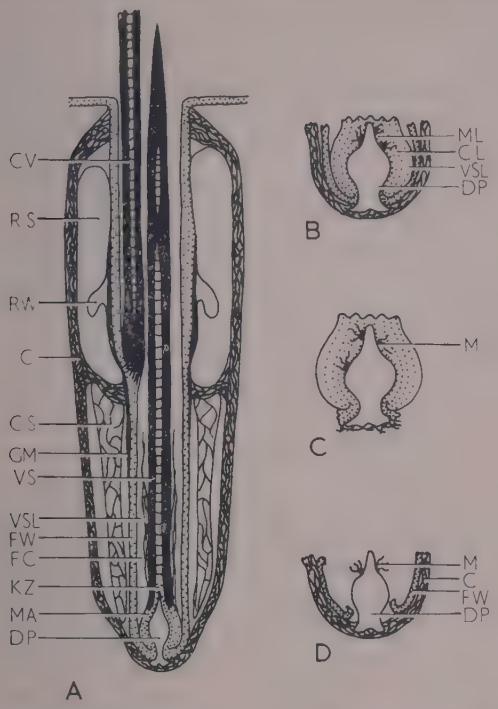
White-haired red-eyed guinea-pigs and Hooded rats were used. All transplants in these series were autografts, performed on animals anaesthetised by intracardial injection of Nembutal (Abbott), 0·055 c.c./100 g. body-weight.

As the follicle is relatively much deeper and narrower than a feather follicle, it is impractical to approach the papilla by incising down the length of the follicle as is the practice with feather follicles. Instead the inner ends of the vibrissa follicles were exposed as follows: the upper lip and the bases of the whiskers were moistened with spirit and a shallow incision about 1–1½ cm. in length was made just dorsal and parallel to the upper lip on one side. The deeper fascia was then cut parallel to the skin surface, the flap of tissue was reflected over a glass rod, and the exposed follicle bases were dissected *in situ* under a low power (×25) stereoscopic microscope, the ends of the follicles being dissected free of loose connective tissue and removed by a transverse cut. These were kept in saline until sufficient (up to 20) had been removed and then the flap was stitched back into place. Such implants will be called 'end bulbs'. The wound healed in 5–8 days, and at no time did the animals have difficulty in feeding or drinking.

These end bulbs (Plate 1, fig. B) were removed by a transverse cut across the proximal end of the perifollicular sinus. In most cases this cut passed through the apex of the papilla just proximal to the keratogenous zone (see Text-fig. 1 A, B), but considerable variation was found; in some cases part of the keratogenous zone of the whisker shaft was included, while in others the cut exposed the apex of the dermal papilla.

The end bulbs were in most cases further dissected:

(i) The whole papilla (Plate 1, fig. C; Text-fig. 1C), i.e. the dermal papilla with its ectodermal investment ('matrix'), was dissected free of adherent follicle wall and capsule by inserting one blade of a pair of extra fine iridectomy scissors into the follicular cavity and making a longitudinal incision almost to the base of the



TEXT-FIG. 1. A, diagrammatic sagittal section of whole whisker follicle (compare Plate 1, figs. A, B). B, diagrammatic sagittal section of 'whole follicle base' (compare Plate, 1, fig. B). C, diagrammatic sagittal section of 'whole papilla' (compare Plate 1, fig. C). D, diagrammatic sagittal section of first stage in obtaining dermal papilla. The ectodermal part of the papilla has been expressed from the follicle base. Some melanocytes remain on the dermal surface (Plate 1, fig. D).

C, capsule; CL, cortical layer; CS, cavernous sinus; CV, club vibrissa; DP, dermal papilla; FC, follicular cavity; FW, follicle wall; GM, glassy membrane; KZ, keratogenous zone; M, melanocytes; MA, matrix; ML, medullary layer; RS, ring sinus; RW, *ringwulst*; VS, vibrissa shaft; VSL, vibrissa sheath layer.

follicle wall. A cut round the base of the whole papilla left only a disk of capsule adherent below the dermal papilla. This was peeled off with forceps. (The term 'whole papillae' has been used for the corresponding structures in feathers, by Lillie & Wang.)

(ii) The dermal papillae (Plate 1, fig. D) were obtained by exerting gentle pressure on the sides of the end bulb with forceps; this pressure causes the dermal papilla and adherent capsule to separate from the ectodermal component of the whisker papilla—the break occurring as an annulus where the follicle wall is

reflected around the base of the dermal papilla (see Text-fig. 1D). In end bulbs which were producing pigmented whiskers, the dermal papilla so removed (Plate 1, fig. D) usually had some melanocytes around its distal surface—many of these had processes which seemed to have been drawn from between the distal ectodermal cells. Most of the melanocytes, however, remained in the ectoderm.

In addition, several whole whisker follicles were dissected out and examined histologically. These preparations were compared with dissections of end bulbs, whole and dermal papillae, and with sections of these structures (Plate 1, figs. A–D). The sections of rat and guinea-pig follicles revealed a close similarity to those of the mouse described by Melaragno & Montagna (1953) and Davidson & Hardy (1952), and confirm the anatomical observations of Vincent (1913). Text-fig. 1A illustrates the anatomy of the whole follicle, and Text-figs. 1B, C are interpretations from this sectioned material.

End bulbs, whole papillae, and dermal papillae were implanted into various sites. Initially the implantation was made into thigh skin which had been shaved and washed with spirit, but later the grafts were implanted into stomach wall, under splenic capsule, and into dorsal ear skin. In all the rats white whisker tissue was always implanted into left ear and black whisker tissue into right ear.

EXPERIMENTAL RESULTS

(*A*) *Implantation of end bulbs*

(i) Twenty-two end bulbs were implanted into shallow slits in the skin over the inner aspect of the thighs of 6 rats and 2 guinea-pigs. Biopsy at intervals of up to 24 weeks showed only degenerative changes, and no production of whisker. All appeared to have become encapsulated by 12 weeks, and later biopsies showed only the remains of the collagenous follicle sheath and fragments of keratin. No vascularization of the implants could be observed.

(ii) Six end bulbs were implanted into the stomach wall of one rat. These had similarly regressed at 14 weeks.

(iii) Acting on a suggestion by Professor H. B. Chase, that early vascularization was probably essential to such implants, 16 end bulbs were implanted under the splenic capsules of 3 rats. Two of these were killed 9 weeks after the operation and the spleens were sectioned. Of 12 end bulbs implanted, 7 were located in the sections and found to be healthy although producing no whisker. The third rat, killed at 13 weeks, had received 4 end bulbs of which 3 were located. These had produced whiskers of 0·5 mm., 1·2 mm., and 1·3 mm. in length.

(iv) Because of the difficulties inherent in the transplantation to spleen, and the impossibility of continuous observation, 14 end bulbs were transplanted to sites under the ear epidermis of 3 guinea-pigs. The ear was shaved and washed with spirit and a small nick made in the dorsum. A small scalpel blade was then inserted parallel to the surface and the pocket so formed was washed out with saline. Precise orientation of the end bulbs was impossible. At biopsy 8–12 weeks

after the operation only 6 of the end bulbs were located. Two of these had produced whisker shafts with estimated lengths 0·6 mm. and 2·8 mm. This last is illustrated in Plate 1, fig. E.

(v) Fifty-two end bulbs were then similarly transplanted into the ears of 7 rats. Six of these animals produced, over a period of 2 years, lengths of recognizable whisker above the skin surface. Many whiskers were also produced which failed to emerge but coiled in the skin and were recognized as elevated 'rings'; several of these were dissected (Plate 2, fig. J). A total of 26 emergent whiskers was produced by these implants, and at least 18 more failed to pierce the skin. This latter number is doubtful, as successive generations, lying together, could not be distinguished from each other in each 'ring': three of the 'rings' which were dissected had in fact two separate whisker coils, and one had three. It is of interest that in one of the double 'rings' the two whiskers coiled in opposite directions although both had apparently been produced from the same implant. One rat, which had received 2 end bulbs which had previously produced black whiskers, produced the following whiskers, all from the same follicle, in the sequence shown: (*a*) A coiled black whisker with truncated tip, about 2 cm. in length. All succeeding whiskers had attenuated tips. (*b*), (*c*), (*d*), (*e*) White whiskers, apparently normal, all over 2 cm. in length; (*b*) is illustrated in Plate 2, fig. G. (*f*) Another small black whisker, definitely from the same follicle, 1·2 cm. long. (*g*) A small hair-like whisker, dark at the tip with a white shaft. The rat at this time was nearly 2 years old, and showed considerable hair-loss and depigmentation of its pelage. It died with this whisker still present and the ear was sectioned. Unfortunately this was technically unsuccessful. The follicle may be seen, but fixation distortion has resulted in considerable damage to the whisker base and the papilla. Such pigmentation changes in successive generations occurred in several series.

(*B*) *Implantation of whole papillae*

(i) One guinea-pig produced 2 successive whiskers from one follicle after 12 whole papillae had been implanted into its left ear. Four additional papillae, apparently normal but with no whisker or follicular structures, were found at biopsy. Another guinea-pig received 3 whole papillae, produced no whisker, and no implants could be found at biopsy 8 months later. A guinea-pig which had received 3 whole papillae died 6 days later. The implants had achieved a blood-supply but had produced no whisker.

(ii) Sixty-two whole papillae were implanted into the ears of 6 rats. Four of these rats produced emergent whiskers, one produced only 'rings', and one produced nothing and no traces of implants could be found at biopsy 13 months later. All whiskers were of donor-type pigmentation (13 white in left ears, 12 black in right ears) except for one, third generation in right ear, which had a black tip but a white shaft. Two second-generation white whiskers are shown

in Plate 2, fig. H. Five successive generations were produced from one follicle, all apparently normal whiskers.

There was no apparent difference in size between whiskers produced from whole papillae and from end bulbs.

Sections of rat ears in which whole papillae had been implanted and which were producing whisker, revealed that the follicles organized by the implants were of two kinds. In several cases the follicular epithelium was very thick and resembled that of the whisker follicle (Plate 2, fig. K); in most cases, however, it was thin and resembled that of local follicles (Plate 2, fig. L). In two cases it is possible to observe a discontinuity at a level in the follicle, the epithelium being much thicker (about 4 cells) proximally and thin (1–2 cells) distally. Sebaceous glands attached to thin epithelium resemble those of the local hairs, but those on the thicker-walled follicles, while not resembling those of the whisker in their position and relationship with a peripheral blood-sinus, do resemble them in that they appear to be ductless, and poorly developed in general. In no case was a peripheral blood-sinus found, nor a *ringwulst*, nor a glassy membrane. These latter observations apply also to end-bulb implants; however, all of these which were sectioned had whisker-type follicles in all other respects (Plate 2, fig. K).

(*C*) *Implantation of dermal papillae*

Sections of one rat ear which had received 11 dermal papillae, 4 weeks after the operation, revealed no necrotic or otherwise degenerate structures, but no whisker had been produced (as judged by the diameter and the character of the medullae of such hairs as were present). Several aggregations of cells in the mesoderm were tentatively identified as the implants. One of these was in contiguity with a fold of ectoderm from an adjacent follicle; unfortunately the contiguity is in the plane of the section and cannot be photographed. Two follicles in the vicinity have very short lengths of hair and no sebaceous glands, and the apices of the papillae are directed toward the cartilage and their bases toward the skin. It is perhaps equally likely that these follicles have not been organized by the implants but have been broken and re-orientated by the actual implantation procedure and subsequent reorganization of the superficial layers. Their dermal papillae are not abnormally large as compared with others near by, and are small compared with those of the whiskers.

A further two rats each received 10 dermal papillae, and neither produced any recognizable whisker in periods of 8 and 9 months after the operation. 'Inverted' follicle bases may be seen in section.

Thirteen dermal papillae, selected for large size, were implanted into a small area (about 2 sq. mm.) of the ear of another rat; the implantation site was marked with Indian ink on the surface after the scab had fallen off (3 days post operation). The rat was killed 7 days after the operation and the site sectioned. Seven dermal papillae have been located with certainty. Despite some difficulty in adequate preparation and staining of these sections it may be clearly seen that

epidermal downgrowths have appeared which have come into relationship with the dermal papillae. Three separate downgrowths have been examined in detail. One appears to derive from the original cut edge of the incision, one from a local hair follicle cut during the incision, and one may have derived directly from the surface epidermis. (It is not clear whether this epidermis represents the original ear surface or whether the original flap died and the site was covered by growth from the margins.) Plate 2, fig. M illustrates part of one section of the site; two downgrowths may be seen, one of which has invested three implanted dermal papillae, which have been sectioned tangentially. The section was chosen as it shows the connexion with the epidermis. Plate 2, fig. N shows part of another section (80μ distant); a dermal papilla has acquired a 'matrix' from the downgrowth.

(*D*) *Structure of whiskers produced by the implants*

Those whiskers which have been produced from the implants have all been small compared with natural whiskers, and many have been more or less curled, a condition which is only found very rarely in the normal whisker. In some cases the first-generation whisker from an implant has appeared truncated at its tip, but most of these, and all whiskers of subsequent generations, have tapered to a fine tip. The rate of growth has not been measured accurately, but appears to be very much slower than whiskers growing in their own follicles. Maturity of the whiskers was attained in all but a few cases, the shed whisker having a 'club' end which compares exactly with those of naturally shed whiskers. However, at no time were two whiskers present in one 'follicle', and a period of at least a month elapsed between the shedding of one generation and the emergence of the tip of the next. Sections of the ears which had received implants and were producing whiskers suggested that the 'follicles' in which these whiskers grew were formed from both local ectoderm and donor ectoderm—this is doubtful in cases where whole papillae were implanted, where it seemed as if the entire follicle was derived from local ectoderm (see above under (*B*) (ii)). In no case could any ring sinus or capsule be distinguished. This may account for the fact that when attempts were made to dissect or pluck out these whiskers, the papilla almost invariably came out on the end of the shaft.

(*E*) *Effect of removal of whisker bases on subsequent growth*

In all cases whisker bases were dissected only from one side of the animal. A smaller number of whiskers was subsequently produced on this side, and some evidence for the belief that follicles whose papillae have been removed degenerate has been obtained by examining the site of removal at autopsy, when cords of fibrous tissue normal to the surface were found; these are not present before the operation, and may represent degenerated capsules or perhaps follicles. Their number corresponds approximately with the number of follicle end bulbs removed.

DISCUSSION

These experiments have shown conclusively that whole follicle end bulbs and isolated whole whisker papillae (containing dermal and epidermal components) remain viable and may produce whiskers in a new site. The whiskers are shed and new whisker grows from the same follicle. However, a large proportion of transplants failed. In many cases histological examination indicated not only that some of the implants had regressed, but that others had been lost entirely, presumably due to imperfections of the implantation procedure. For this reason it is considered that the low percentage of successful transplants does not necessarily reflect a low viability of the implants, but rather an inadequate preparation of the implantation sites.

The fact that several of the implants have produced more than one whisker generation must mean that it is not only that the whisker which was being produced before removal is completed, but that new cycles are initiated in the new site. The papilla must therefore retain its integrity (as does the feather papilla) and be unaffected by the reorganization of the follicle during the growth cycle. This reorganization in vibrissa follicles is by no means as dramatic as in some hair follicles, but the close relationship of the two structures makes it extremely likely that the hair papilla is also permanent in this sense.

The orientation of the whiskers produced by the implants has been completely random, and presumably derives from the orientation of the implanted papilla rather than from any local influence; this would agree with the results of Lillie and Wang on the feather.

Those experiments in which dermal papillae were implanted have demonstrated that the vibrissa dermal papilla, like the feather papilla, retains its inductive capacity and, even in the adult, may call upon local ectoderm to invest it. The new follicles organized by the implanted papillae are not recognizable among the native follicles 4 weeks after the operation (except perhaps in their orientation, which may well depend upon that of the implant). It is unlikely that they have degenerated, as none of the remains were found. It may therefore be supposed that hair and follicles of local type are produced by the local ectoderm under the influence of a vibrissa dermal papilla. The vibrissa whole papilla, on the other hand, produces hair of donor type (whiskers) and part of the follicle often appears to be of donor ectoderm. This lends support to the belief that the differences which result in the production of hair or whisker reside in the *ectoderm* concerned in the production.

The vibrissa dermal papilla is usually much larger than that of the ear hairs. However, 4 weeks after implantation such large dermal papillae cannot be found. The possibility therefore exists that (as in the work of van Scott & Ekel) an ectodermal influence on the papilla has resulted in a reduction of the volume of the dermal papilla and perhaps a corresponding reduction in the number of cells in it. Work is proceeding which will test this hypothesis.

It is concluded that the results published here show a similarity between the epigenetic situation described for feather follicles and that found in whisker and hair follicles; the dermal papilla is a non-specific organizer throughout its history, the local specificity of plumage and probably also pelage being dependent upon intrinsic properties of the local ectoderm.

SUMMARY

1. A method is described for the removal and transplantation of vibrissa follicle bases (end bulbs).

2. The whole papilla may be dissected from the capsule and follicle wall, and the dermal papillae may also be dissected out.

3. Transplants have been made to various sites. No whisker was produced from end bulbs implanted into body skin, but whiskers were produced from end bulbs in the spleen, and from both end bulbs and whole papillae under ear skin.

4. Several generations of whisker have been produced from some of the implants, but retention of the club whisker has not been observed.

5. It is concluded that the whisker papilla is a permanent entity in the same sense as is the feather papilla, despite follicular changes during the growth-cycle.

6. Dermal papillae may cause local ear epidermis to invest them, and presumably this produces hair of local kind, as no whisker or whisker follicle was discovered.

7. It is suggested that the whisker papilla is a non-specific organizer, specificity residing in the ectoderm concerned.

RÉSUMÉ

Transplantation de papilles de vibrisses individuelles de Rat et de Cobaye

1. On décrit une méthode d'exérèse et de transplantation des follicules basaux des vibrisses (bulbes terminaux).

2. On peut disséquer la papille entière à partir de la capsule et de la paroi folliculaire, et les papilles dermiques peuvent également être extraites par dissection.

3. Les transplantations ont été faites en divers endroits. Les bulbes terminaux implantés dans la peau du corps n'ont pas produit de vibrisses, mais celles-ci se sont formées à partir de bulbes implantés dans la rate et à partir de bulbes et de papilles entières transplantés sous la peau de l'oreille.

4. Quelques implants ont donné plusieurs générations de vibrisses, mais on n'a pas observé de rétention de vibrisse à croissance achevée.

5. On conclut que la papille de la vibrisse est une entité permanente comme l'est la papille plumaire, malgré les modifications folliculaires au cours du cycle de croissance.

6. Les papilles dermiques peuvent induire l'épiderme local de l'oreille à les

revêtir, et ceci donne probablement naissance à du poil de type local, car on n'a pas observé de vibrisse ou de follicule correspondant.

7. La papille de vibrisse serait un organisateur non spécifique, la spécificité étant localisée dans l'ectoderme mis en jeu.

ACKNOWLEDGEMENTS

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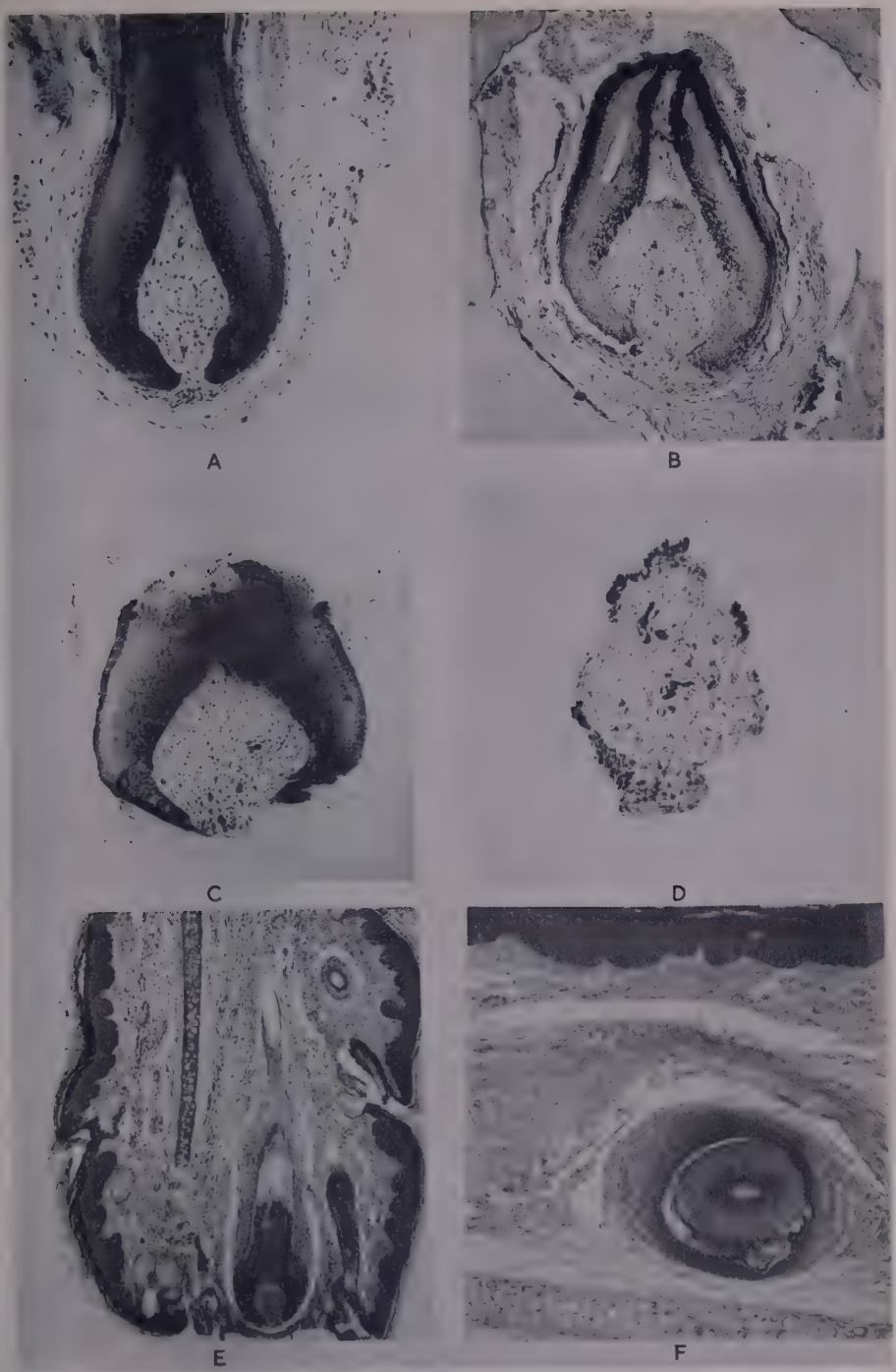
EXPLANATION OF PLATES

Plate 1

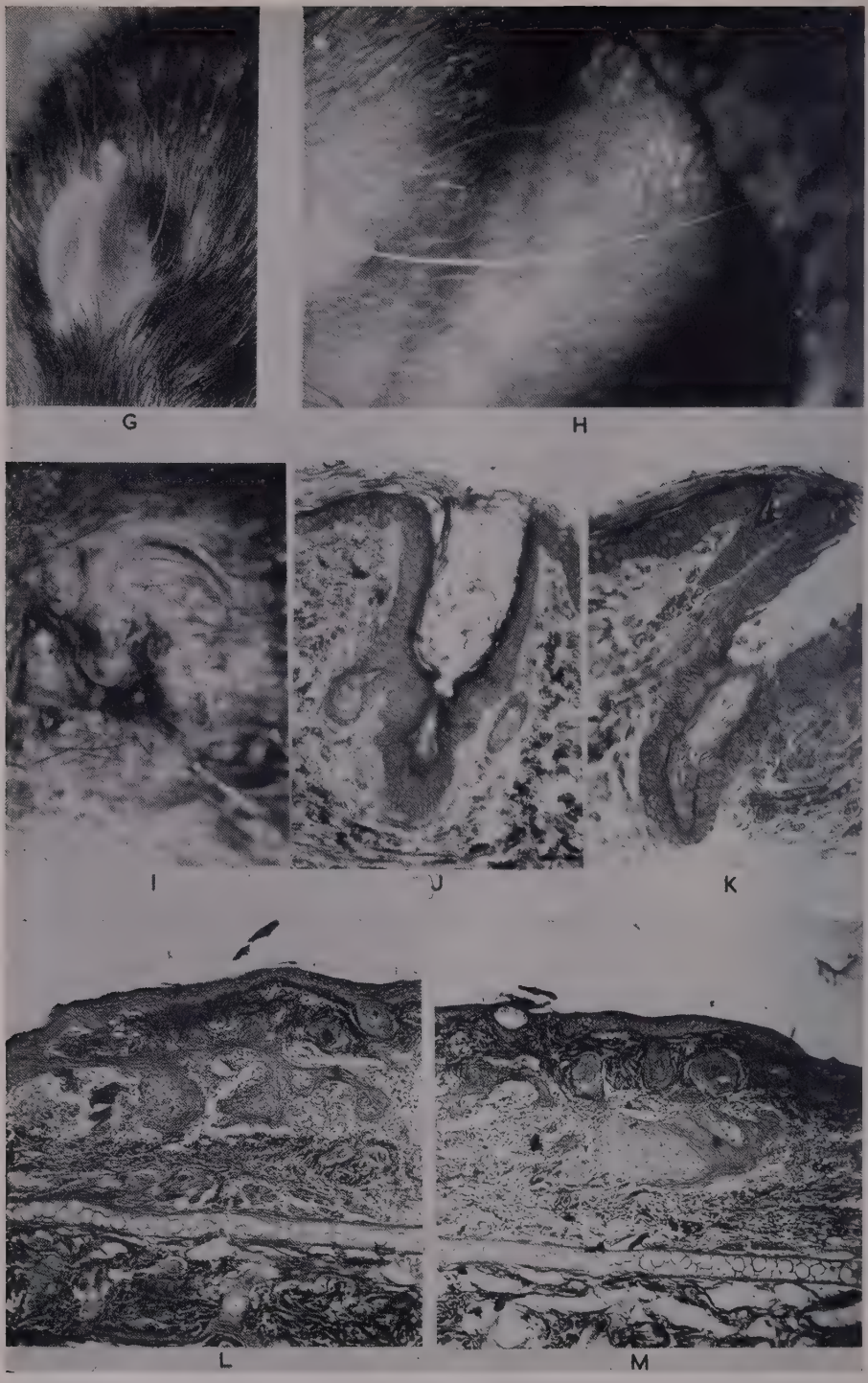
Fig. A. Longitudinal section of proximal end of Hooded rat whisker follicle, sagittal; stained Harris's haematoxylin and eosin. × 190.

Fig. B. Longitudinal section of rat follicle 'end bulb', sagittal; stained by Mallory's 1936 method. This tissue was orientated and sectioned in a plasma clot, which may be seen surrounding it. × 210.



J. COHEN

Plate 1



J. COHEN

Plate 2

FIG. C. Longitudinal section of rat 'whole papilla', parasagittal; stained Harris's haematoxylin and eosin. The material surrounding it is gelatine, in which the papilla was orientated and sectioned. × 210.

FIG. D. Longitudinal section of a 'dermal papilla', dissected routinely from a rat-whisker follicle base, parasagittal; stained by Mallory's 1936 method. This tissue shrinks considerably in preparation. Melanocytes may be seen around the distal end. The cellular tissue at the left of the base is liver, on which it was orientated and sectioned. × 450.

FIG. E. Section of guinea-pig ear normal to surface, showing implanted whole follicle base which has produced follicular structures and a length of whisker, not all of which is in the plane of the section. Stained by Harris' haematoxylin and eosin. × 35.

FIG. F. A similar section of guinea-pig ear, with a whisker in transverse section; stained by Mallory's 1936 method. × 75.

PLATE 2

FIG. G. Right ear of Hooded Rat which has received whole follicle bases (black). The whisker shown is second generation, white, and apparently structurally normal.

FIG. H. Second generation white whiskers produced in Hooded Rat left ear which had received whole papillae (white).

FIG. J. Dissection of a 'ring' produced after implantation of 'whole papillae' (white) into the left ear of a Hooded Rat. The white whisker and its follicle may be seen.

FIG. K. Section of rat ear through the distal part of a follicle associated with an implanted end bulb and the whiskers it produced. The follicle wall is thick, like that of the distal part of the normal whisker follicle, and the sebaceous glands are typically whisker type. Stained Mallory's 1936 method. × 75.

FIG. L. Section of rat ear through the distal part of a follicle associated with an implanted whole papilla and the whiskers it produced. The follicle wall is thin like that of local follicles. A club whisker base is present. Unfortunately the section is not flat and parts are not in focus. Stained Mallory's 1936 method. × 15.

FIG. M. Section of rat ear, normal to surface, 7 days after implantation of dermal papillae. An epidermal downgrowth (from the left of the picture) may be seen to have come into association with three dermal papillae, which have been sectioned tangentially. This section was chosen despite its technical imperfections as it shows the connexion of the downgrowth with the epidermis (the original incision was to the *right* of the photograph). Stained Mallory's 1936 method. × 40.

FIG. N. Another section from the same series, 80 μ distant. Another implanted dermal papilla may be seen to have acquired a 'matrix'. × 50.

(*Manuscript received 1 : viii : 60*)

The Development of the Phagocytic Activity of the Reticulo-endothelial System in the Chick

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WITH TWO PLATES

INTRODUCTION

In the vertebrates the process of phagocytosis is one of the well-recognized defence mechanisms of the body and by this means foreign particulate matter is removed from any tissue to which it has gained access. In birds and mammals the liver, spleen, and bone-marrow are particularly active centres of this phagocytic process. The liver and spleen are of particular importance, for not only do they possess an abundance of cells capable of phagocytosis but, according to some authors (Irwin, 1932; Easton, 1952), to them are transported through the blood system various wandering cells, 'macrophages', which have ingested foreign particulate matter in other situations.

The extensive uptake of particulate matter is part of the function of the reticulo-endothelial system. Little appears to be known of the development of this activity in ontogeny, though there are observations on phagocytosis in general in the embryo.

Phagocytosis in the chick embryo *in vivo* was recorded by Kiyono (1918), who found that particles of dye were taken up by the 'proliferating tissue cells' of chick blastoderms, and by the embryonal blood-cells in older embryos. Dabrowska (1950) has reviewed very adequately the work of Heine (1936) and Steinmüller (1937), both of whom found phagocytic properties in all the types of cells in early blastoderms, including the red blood corpuscles. Dabrowska (1950), using more accurate methods, found that phagocytosis took place in ectodermal, mesodermal, and endodermal cells of early blastoderms but not in the red blood corpuscles. She agreed with the view of Heine (1936) that the phagocytic properties of the ectodermal and endodermal cells disappeared at about 2 to 3 days of incubation. Dabrowska noted as exceptions cells of the amnion and certain epithelia which did not lose their phagocytic properties until 10 to 15 days of incubation; after this time only migratory cells, white blood corpuscles, and the peritoneal epithelium retained the ability to phagocytize up to the time of hatching. Perez del Castillo (1957), using a carbon suspension as an indicator of phagocytic activity in the liver, concluded that phagocytosis

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does not take place at all before the 12th day of incubation and even then not markedly until the 16th day.

In experiments on chick blastoderms *in vitro*, in which particulate matter was used to mark cells for the study of morphogenetic movements, Jacobson (1938) and later Spratt (1946) observed that epiblast cells which were on the point of invaginating at the primitive groove to form the mesoderm were able to phagocytize particles of carbon placed upon their surface. The assumption of this phagocytic function by the invaginating epiblast cells was later correlated by Rudnick (1944) with the loss of the epithelial characters, in particular coherence, of the epiblast cells as they invaginate to form the mesoderm.

MATERIALS AND METHODS

Thorotrast, a colloidal suspension of 25 per cent. thorium dioxide and 25 per cent. aqueous dextrin by volume, was used as particulate material for injection, for not only can it be detected by observation in tissue sections but concentrations of it can be detected by radiographic methods. It was originally used in hepato-lienography of man (Radt, 1930; Volicer, 1931) and has been used for studies in some ways similar to those reported here by Foxon & Rowson (1956), who used it to demonstrate phagocytic activity in frogs. The particles in Thorotrast are reported to have a mean diameter of the order of 10^{-6} cm., that is, 10 Å (Maxfield & Mortensen, 1941). Irwin (1932) has shown that after 5–10 minutes in circulating blood the particles tend to aggregate until visible aggregations are formed. It is at present uncertain whether there is any difference in the process by which these particles of various sizes enter the cells. The author estimates that Thorotrast contains about 160 mg. thorium dioxide per ml. A 0·22 per cent. colloidal suspension of silver (Ag^{110}) in water, with added gelatin, glucose, and NaOH, has also been used for some experiments. The particle size is between 10 and 50 mμ (measured with an electron microscope).

The colloids were administered by injection. Embryos of 6 days' incubation and over, in which the allantoic vein was well developed, were injected by the procedure first described by Polk, Buddingh, & Goodpasture (1938) and modified by Beveridge & Burnet (1946). Using a toothed-disk attachment to a dental drill a small rectangle of shell (0·5 × 1 cm.) was cut from above a suitable part of the allantoic vein, the position of which had previously been determined by 'candling'. The underlying shell membrane was rendered transparent by placing on it a drop of liquid paraffin so that the vein could be seen. By means of a 30-gauge hypodermic needle and a 1-ml. tuberculin syringe Thorotrast was injected into the vein. The hole in the shell was sealed with a drop of hot embedding wax.

Embryos of under 6 days' incubation were injected as follows. A half-inch square of shell was cut above the embryo and removed together with the shell membrane to expose the embryo. Under a binocular microscope a very small, unmeasured quantity of Thorotrast was injected into a tributary of the vitelline vein by means of a glass micropipette held in a 'Singer' micromanipulator.

The vessel was then sealed by cautery using a hot needle, thus preventing leakage of blood and Thorotrast. The egg was temporarily sealed with a small square of transparent foil to prevent evaporation.

Incubation was continued after injection for periods ranging from $1\frac{3}{4}$ hours to several days, during which time some of the older embryos hatched. After a known time each embryo which was to be examined was removed from the egg together with a small piece of yolk-sac wall and fixed in formal-saline. Sections were cut at 8 μ, and stained with Ehrlich's haematoxylin and counter-stained with eosin.

Some of the embryos injected with Thorotrast by way of the allantoic vein at 14–20 days' incubation were allowed to hatch and were examined for Thorotrast at varying periods after hatching. They were examined first by radiography, which showed the position of any considerable concentration of Thorotrast, and sections were then cut and stained using the methods described above. For one experiment two embryos of 17 days' incubation were injected with 0·05 ml. radioactive colloidal silver (Ag^{110}). These were allowed to hatch and their faeces were collected and the radio-activity determined in a well-type scintillation counter.

For comparison a hatched chicken ($1\frac{1}{2}$ months old) was injected with 0·1 ml. Thorotrast by way of the pectoral vein, and was killed and examined for Thorotrast after 24 hours.

Thorotrast has been identified in the tissues of the chick sections by two methods. In large amounts Thorotrast is visible in the form of deposits which have a distinctive green–blue sheen when observed under an ordinary light microscope. However, small amounts of Thorotrast in individual macrophages are often invisible by this method, but they can be seen by oblique transmitted illumination, which gives a dark-ground effect, as used by Faber (1937); it can conveniently be produced with a phase-contrast microscope as described by Baxter (1960).

RESULTS

Embryos of 3–6 days' incubation at injection

Table 1 shows the treatment received by the embryos of this group and the organs in which Thorotrast was found to be taken up. It may at once be seen that relatively little uptake of Thorotrast takes place in the embryo proper before the organs associated with the reticulo-endothelial system are differentiated.

In the liver, Thorotrast may be seen in the Kupffer cells lining the sinusoids in embryos of only 4 days' incubation, that is, on the same day as the liver starts to differentiate from the gut (Plate 1, figs. E, F). In fig. G of Plate 2 the presence of Thorotrast in the Kupffer cells gives them a characteristic 'bubbly' or alveolar appearance.

In the spleen the Thorotrast is taken up at 5 days' incubation to a lesser extent

than in the liver, and is seen in macrophages which are scattered throughout the organ (Plate 1, figs. C, D).

TABLE 1

The occurrence of Thorotrast in the organs of chick embryos injected at 6 days' incubation or less

No. of days of incubation when injected	*Time in hours between injection and fixation*	*Organs examined for Thorotrast*					
		Yolk-sac wall	*Areolar connective tissue of mesenteries and body-wall*	*Gut*	*Kidney*	*Liver*	*Spleen*
3	1¾	T	—	—	—	ND	ND
3	3	T	—	—	—	ND	ND
3	5	T	T	T	—	ND	ND
4	3	T	—	—	T	T	ND
4	3	T	—	—	T	T	ND
5	3	T	T	—	T	T	T
5	5	T	—	—	T	T	T
6	3½	T	T	T	T	T	T
6	5	T	T	T	T	T	T

T, Thorotrast present; —, no Thorotrast present; ND, organ not yet developed.

The yolk-sac wall was found to be of some importance in the uptake of Thorotrast, which may be seen to be taken up by macrophages in the mesodermal part of the wall from the earliest times of injection (Plate 2, figs. H, I, J). In Plate 2, fig. H the loaded macrophages assume the characteristic 'bubbly' appearance as described in the Kupffer cells.

TABLE 2

The occurrence of Thorotrast in the organs of chick embryos injected with 0·1 ml. at 6 days' incubation and over

No. of days of incubation when injected	*Time in hours between injection and fixation*	*Organs examined for Thorotrast*					
		Yolk-sac wall	*Areolar connective tissue of mesenteries and body-wall*	*Gut*	*Kidney*	*Liver*	*Spleen*
6	2	T	—	—	T	T	—
7	24	T	—	—	T	T	T
8	2	T	T	—	T	T	T
8	3	T	—	—	—	T	T
8	20	T	—	—	—	T	T
8	24	T	T	—	T	T	T
9	24	T	T	T	T	T	T
9	24	T	—	—	T	T	T

T, Thorotrast present; —, no Thorotrast present.

The accumulation of Thorotrast in the kidneys occurs in the glomeruli, where large deposits of Thorotrast may be seen (Plate 1, fig. B) which on closer examination appear to be adhering to the capillary walls of the glomeruli. A few Thorotrast-laden macrophages may also be seen in the kidney tissue.

In the areolar connective tissue of the mesenteries and body-wall and in the gut-wall Thorotrast uptake occurs in a few scattered macrophages. The extent of uptake is nothing like that of the other organs mentioned.

Embryos of 6–9 days' incubation at injection

Table 2 shows the treatment received by the embryos of this group and the organs in which Thorotrast was found to be taken up. Thorotrast uptake in this group was much the same as that already described in the previous section. The importance of the yolk-sac wall as a site of Thorotrast uptake diminished as the liver and spleen and other organs enlarged, although it continued to take up Thorotrast until the chick hatched.

Embryos of 14–19 days' incubation at injection, subsequently allowed to hatch

Table 3 shows the treatment received by the embryos of this group and the organs in which Thorotrast was found to be taken up. The uptake was as described for group (*b*). In the two specimens injected at 14 days the lungs and bone-marrow were also examined: Thorotrast was present but in very small quantities in comparison with the liver and spleen.

TABLE 3

The occurrence of Thorotrast in the organs of chicks which were injected before hatching

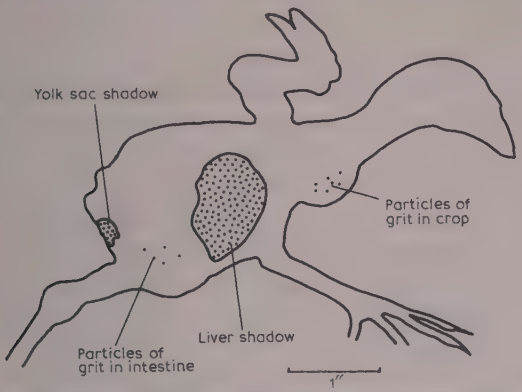
No. of days of incubation when injected	*Dose in ml.*	*Time in days between injection and fixation*	*Organs examined for Thorotrast*							
			Yolk-sac wall	*Areolar connective tissue of mesenteries and body-wall*	*Gut*	*Kidney*	*Liver*	*Spleen*	*Lung*	*Bone-marrow*
14	0·2	14	T	—	—	—	T	T	T	T
14	0·2	21	—	—	—	—	T	T	T	T
18	0·05	4	T	—	—	—	T	T	NE	NE
18	0·05	4	T	—	—	—	T	T	NE	NE
18	0·05	4	T	—	—	—	T	T	NE	NE
18	0·1	4	T	—	—	—	T	T	NE	NE
19	0·04	7	T	—	—	—	T	T	NE	NE
19	0·05	7	T	—	—	—	T	T	NE	NE

T, Thorotrast present; —, no Thorotrast present; NE, organ not examined.

The uptake of particles in the yolk-sac wall

The fate of the particles taken up in the yolk-sac wall was investigated by taking radiographs of hatched chicks injected with Thorotrast before hatching from 14 to 19 days of incubation. The series of radiographs show an increase

in intensity of the yolk-sac shadow together with diminution in its size up to 6–7 days after hatching (Plate 2, figs. K, L; Text-fig. 1), and after that the disappearance of the shadow as the yolk sac is completely absorbed.



TEXT-FIG. 1. Tracing of a radiograph of a 7-day-old chick injected with 0·1 ml. Thorotrast at 19 days of incubation. The outlines of the liver and the remains of the yolk-sac wall show the main deposits of Thorotrast.

The disappearance of the Thorotrast from the region of the yolk-sac wall could be explained by supposing either that the mesodermal cells could become free from the yolk-sac wall and migrate to the liver or that the cells containing Thorotrast could pass through the endoderm of the yolk sac into the lumen of the gut. It is conceivable that both processes might be at work simultaneously. In the event of the death of a Thorotrast-containing cell, the remains would presumably be ingested by a wandering macrophage and so elimination could still follow one of these two routes. Preliminary observations have shown that colloidal silver is treated in the body in the same way as Thorotrast, and the faeces from hatched chicks which had been injected at 17 days of incubation were therefore collected and examined for radioactivity. The results of this experiment are set out in Table 4. This shows that particles of silver are eliminated through the gut. The source of these particles will be discussed later.

TABLE 4

Analysis of faeces of 2 chicks injected with 0·05 ml. Ag^{110} *at 17 days of incubation. Faeces taken over 3 days, 5 days after hatching*

Chick	*Wt. of faeces produced over 3 days (in g.)*	*Counts per minute of faeces*	*Background*	*Counts per minute per g. faeces*	*Counts per minute of standard 0·0001 ml.* Ag^{110}	*Percentage of injected dose in faeces over 3 days*
1	6	1,853	443	235	972	0·3
2	7·5	3,338	443	386	972	0·6

The counts were taken over a period of 2 minutes and divided by two.

DISCUSSION

The sites of uptake of Thorotrast appear to be the same in the older embryo chick as in the young chicken, with the exception of the yolk-sac wall, which, of course, is not present in a chicken which has been hatched for 1 week or more. The main site of uptake is undoubtedly the liver, but Thorotrast is also filtered from the blood by the kidneys and taken up in smaller quantities by the macrophages of the spleen, lung, bone-marrow, and areolar connective tissue of the mesenteries and body-wall, the latter more often in cases of larger doses.

The demonstrated presence of Thorotrast in the Kupffer cells of the liver of embryos of 4 days' incubation (Plate 1, figs. E, F), in the spleen of embryos of 5 days' incubation (Plate 1, figs. C, D), and in the yolk-sac wall of embryos of 3 days' incubation (Plate 2, figs. I, J) shows that phagocytosis of Thorotrast occurs in the liver and spleen as soon as they differentiate and in the yolk sac as soon as the mesodermal part of the wall is formed. Perez del Castillo (1957), using a carbon suspension as an indicator of phagocytic activity in the liver of chick embryos, concluded that phagocytosis does not occur to any extent in the embryo chick until the 16th day of incubation, and not at all before the 12th day. The present results show that phagocytosis of Thorotrast, at least, takes place at a considerably earlier stage.

The absence of Thorotrast in the body of two of the embryos of under 4 days' incubation in which the liver and spleen were not differentiated would suggest that rapid and extensive uptake of particulate matter is not a property of all undifferentiated mesodermal cells, but that it is a specialized activity which develops in some cells of certain tissues. The work of Jacobson (1938), Dabrowska (1950), and others shows, however, that phagocytosis does take place to a certain extent in all the cell types of the early blastoderm.

The occurrence of radioactivity in the faeces after injection with silver (Ag^{110}) must now be discussed. Two sources seem possible. Firstly, it can be imagined that any particles from the yolk sac pass directly into the lumen of the gut and so to the exterior; but in this connexion it must be remembered that in some animals (Irwin, 1932; Lambin, 1932) it has been claimed that Thorotrast is eliminated from the liver by transport in macrophages to the lungs, thence up the trachea, and so presumably into the alimentary canal, and also (Çsaba, Niedermann & Rappay, 1954) that silver stored in the Kupffer cells is eliminated with the bile through the alimentary canal. Thus it might be that the radioactivity detected in the faeces of the chicks could have been derived by one of these alternative routes. Secondly, the disappearance of the particles from the yolk-sac wall might be due to their transportation to the liver. In sections of the yolk sac from chicks killed 7 days after hatching, and which had been injected with Thorotrast at 19 days of incubation, macrophages with Thorotrast have been seen free in the lumen of the sac. Thus it appears that macrophages do pass into the lumen of the sac, but so far no macrophages have been detected in the

sections through the stalk of the yolk sac, a situation in which it would appear reasonable to expect to find them if this was a main route of elimination. The author does not consider that the results so far obtained allow of any conclusion as to the more likely method of elimination in the chick.

SUMMARY

1. The embryonic development of the ability of some cells to undertake extensive phagocytosis has been studied in the chick embryo using the particulate materials 'Thorotrast' and colloidal silver (Ag^{110}).

2. Extensive phagocytosis of Thorotrast particles by the embryo chick and the young chicken takes place in the liver and spleen and to some extent in the lung, bone-marrow, and areolar connective tissue of the mesenteries and body-wall, uptake in the latter being more evident in cases of larger doses. In addition, the embryo chick takes up Thorotrast in the mesodermal part of the yolk-sac wall.

3. In the embryo chick rapid and extensive uptake of particulate matter does not occur until the main centres of the reticulo-endothelial system have been differentiated.

4. Particles taken up by the yolk-sac wall of the embryo chick disappear after hatching. The exact mechanism involved has not yet been established.

RÉSUMÉ

Développement de l'activité phagocytaire du système réticulo-endothélial chez le Poulet

Le développement de l'aptitude de certaines cellules à assumer une phagocytose étendue a été étudié chez l'embryon de poulet à l'aide de substances particulaires, 'Thorotrast' et argent colloïdal (Ag^{110}). Chez l'embryon et le jeune poulet, une phagocytose importante de particules de 'Thorotrast' a lieu dans le foie et la rate et, dans une certaine mesure, dans le poumon, la moelle osseuse et le tissu conjonctif aréolé des mésentères et de la paroi du corps, l'absorption dans ce dernier cas étant plus nette pour de fortes doses. De plus, chez l'embryon, le 'Thorotrast' est absorbé par le mésoderme de la paroi du sac vitellin.

Chez l'embryon, on n'observe pas d'absorption étendue de substances particulaires avant que se soient différenciés les principaux centres du système réticulo-endothélial. Les particules absorbées par la paroi du sac vitellin disparaissent après l'éclosion. La nature exacte du mécanisme impliqué n'a pas encore été établie.

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EXPLANATION OF PLATES

ABBREVIATIONS: *end.*, endoderm; *g*, gut; *gl*, glomerulus; *k*, kidney; *K*, Kupffer cell; *l*, liver; *ma*, macrophage; *mes*, mesoderm; *s*, sinusoid; *spl*, spleen; *T*, Thorotrast; *y.s.*, yolk sac.

PLATE 1

FIG. A. Eight-day embryo, 24 hours after Thorotrast injection, removed from shell and radiographed on process plate. Thorotrast shadows are visible in the liver and kidneys (*l* and *k*).

FIG. B. T.S. kidney of 8-day embryo, 24 hours after Thorotrast injection. Photographed with phase-contrast dark-ground illumination. Large quantities of Thorotrast may be seen as shining white masses adhering to the walls of the glomerular capillaries, having presumably been filtered out by the passage of blood fluids into the kidney tubules. A few Thorotrast-laden macrophages (*ma*) may also be seen in the kidney tissue.

FIG. C. T.S. spleen of 5-day embryo, 3 hours after Thorotrast injection (light background).

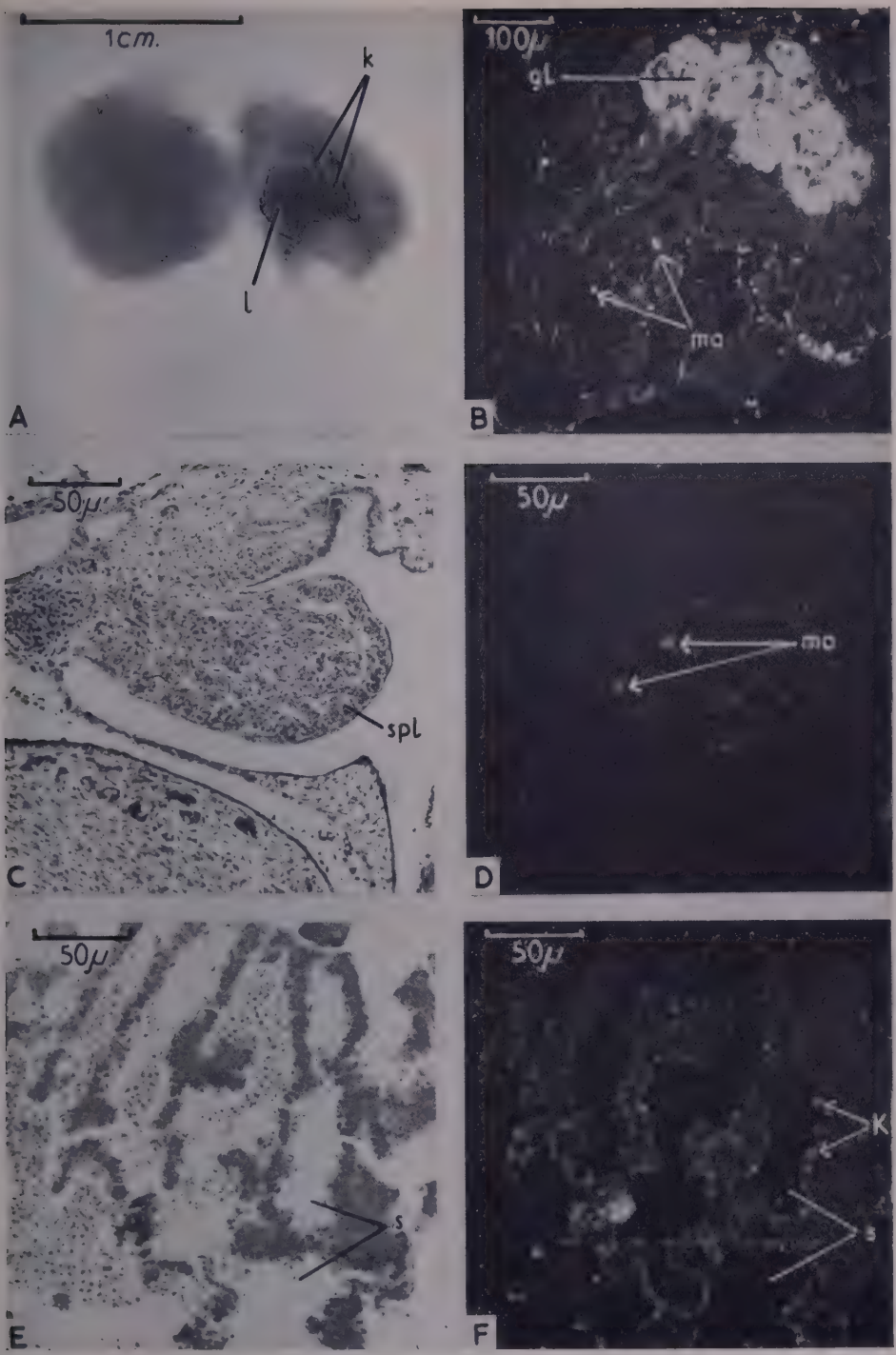

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Plate 1

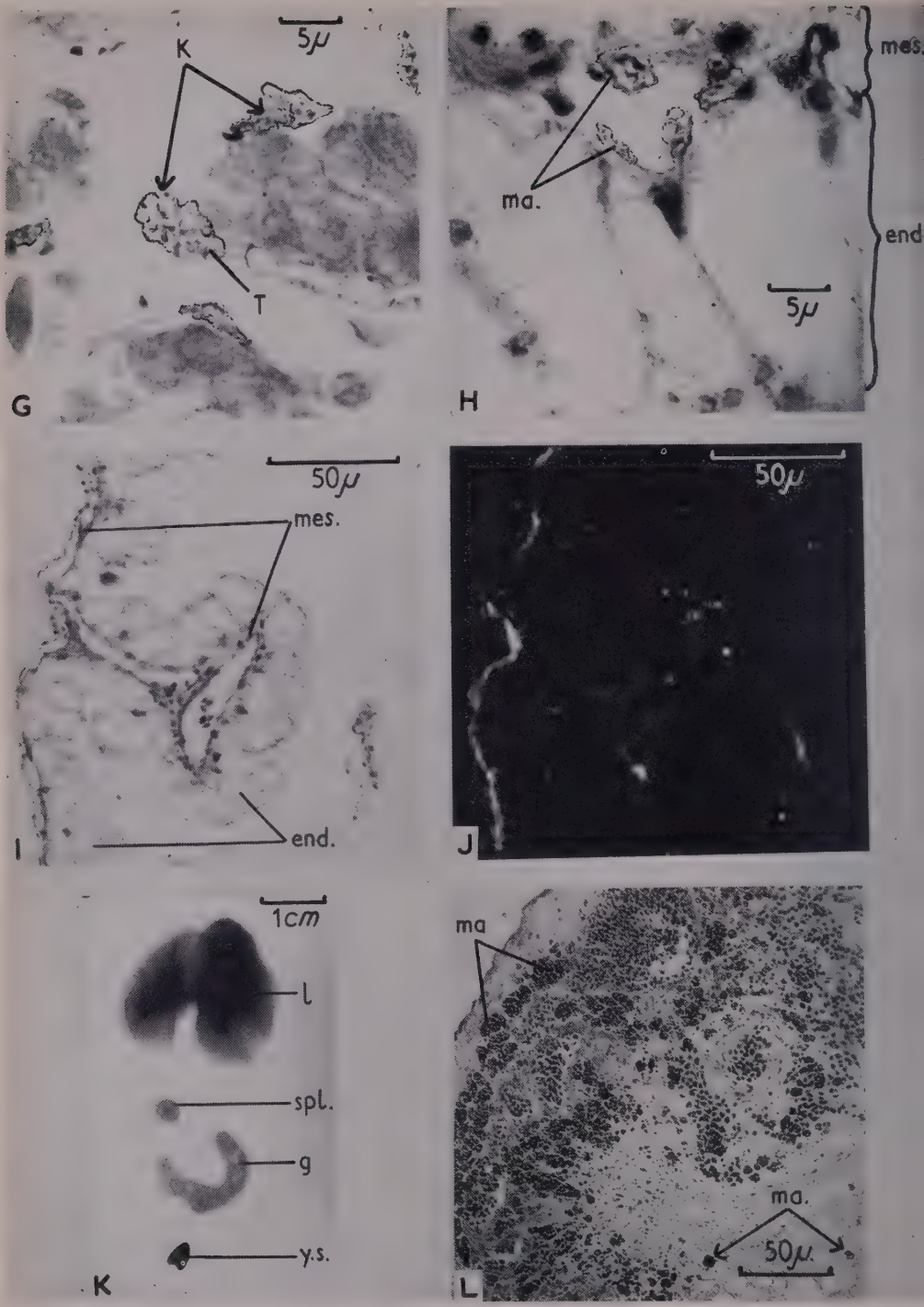

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Plate 2

FIG. D. Identical with fig. C but photographed with dark background as in fig. B. Thorotrast-laden macrophages (*ma*) may now be seen in the spleen tissue.

FIG. E. T.S. liver of 4-day embryo, 3 hours after Thorotrast injection (light background).

FIG. F. Identical with fig. E but with dark-ground illumination as in fig. B. Thorotrast may be seen in the Kupffer cells (*K*) lining the sinusoids (*s*).

PLATE 2

FIG. G. T.S. liver of 5-day embryo, 3 hours after Thorotrast injection (light background, oil-immersion objective). Note agglutinated Thorotrast particles (*T*) inside the Kupffer cells (*K*).

FIG. H. T.S. yolk-sac wall of 3-day embryo, 3 hours after Thorotrast injection (light background, oil-immersion objective), shows the agglutinated Thorotrast particles inside macrophages (*ma*) in the mesodermal layer (*mes*).

FIG. I. T.S. yolk-sac wall of 3-day embryo, 1¾ hours after Thorotrast injection (light background). Compare with fig. J.

FIG. J. Identical with fig. I but photographed with dark-ground illumination as in Plate 1, fig. B. The line of brightness indicates the position of Thorotrast-laden macrophages which, if compared with Plate 1, figs. B & C, are seen to be in the mesodermal part of the yolk-sac wall.

FIG. K. Positive radiograph of liver, spleen, gut, and yolk sac of a 7-day chick, 9 days after Thorotrast injection into the egg. The dense shadows in the liver and yolk sac indicate large deposits of Thorotrast.

FIG. L. T.S. yolk-sac wall of same chick as in fig. K (light background). Thorotrast-laden macrophages (*ma*) are seen to be present in large numbers in the wall of the very much reduced yolk sac. A few macrophages may be seen in the lumen of the yolk sac.

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Topography of the Presumptive Rudiments in the Endoderm of the Anuran Neurula

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WITH ONE PLATE

INTRODUCTION

In previous papers (Nakamura & Tahara, 1953, 1954), evidence was presented on the formation of the anuran stomach and intestines. The first paper demonstrated the origin of the stomach from the posterior part of the fore-gut of the neurula. The second disproved the archenteric origin of the intestine: contrary to Goette's (1875) long-accepted theory, the archenteron was shown to close up throughout the whole length of the mid-gut. The definitive cavity of the intestine is newly formed by the splitting of the mass of yolk cells.

These and more recent findings make it possible to determine the position occupied in the neurula by the materials which form the various endodermal organs. In the present paper, the data are fully reported and a map of the presumptive materials is produced.

MATERIAL AND METHODS

Embryos of a common Japanese frog, *Rana nigromaculata nigromaculata*, were used. This species was preferred to others because of its quick healing and its undisturbed development after the operation.

To stain the endoderm already invaginated an incision was made in the neurula at the place of the presumptive sucker. Through it, small pieces of agar containing neutral red or Nile blue were inserted into the archenteron and put directly upon the part to be marked. The endoderm was stained in a few minutes. When it was necessary to prevent the transmission of the dye to the other walls of the archenteron, the agar was covered with a piece of cellophane. Slight modifications of this procedure were required for staining some special areas; these will be described at the appropriate place.

Most specimens were reared until the digestive organs had formed and were then dissected after fixation with 10 per cent. formalin. Some specimens were sectioned, after fixation in Zenker's solution without acetic acid and dehydration with dioxan, to study microscopically the exact position of the stain.

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ANATOMICAL AND HISTOLOGICAL OBSERVATIONS

Anatomical and histological features of the development of the digestive organs in the present species tally on the whole with the classical description by Goette (1875) of the embryo of *Bombinator igneus*, but there are a few points of considerable difference. It is desirable, therefore, to present a brief sketch of the phenomena in our species, with definitions of the terms to be applied, before describing the results of vital staining.

We begin with the endoderm of the neurula in stage 14 (after Tahara's (1959) table for *R. japonica*). The archenteron may be divided, as was done by Goette, into three parts, i.e. fore-gut, mid-gut, and hind-gut (Text-fig. 1A, B). The fore-gut lies under the brain region of the neural plate and in front of the yolk mass. It expands considerably both laterally and ventrally. It has also an antero-ventral outpocketing just below the transverse neural fold and a postero-ventral outgrowth beneath the anterior end of the yolk mass, the former usually termed the 'oral evagination' and the latter the 'liver diverticulum'. Adjacent to the posterior end of the latter is a remnant of the blastocoel. The partition separating it from the liver diverticulum is formed of a single layer of yolk cells.

The wall of the fore-gut is one cell thick throughout, except at its posterior side. For convenience of description we adopt a sixfold division of the wall—anterior, posterior, dorsal, ventral, right, and left.

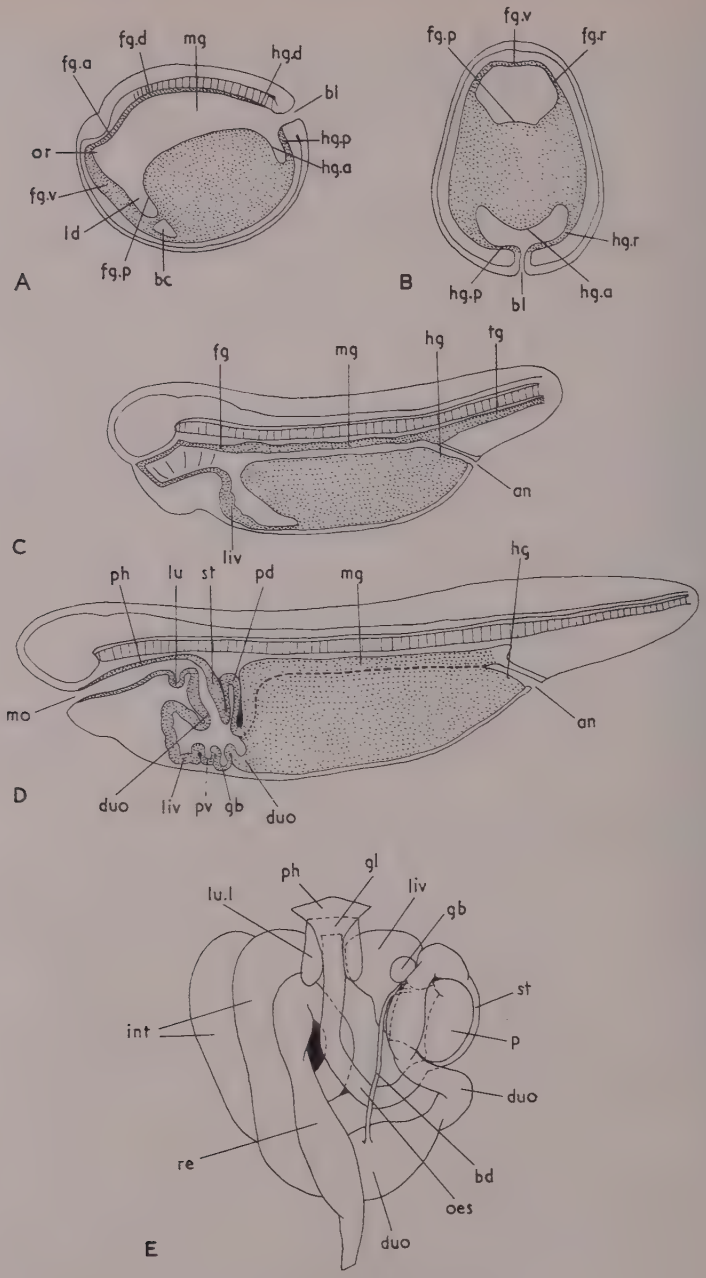
The anterior wall ascends postero-dorsally from the level of the transverse neural fold to that of the middle of the brain region. Posteriorly to it, the dorsal wall (the roof) lies horizontally beneath the hindbrain. On the other hand, the ventral wall (the floor) descends from the antero-dorsal edge of the oral evagination to the posterior end of the liver diverticulum. The posterior wall is identical with the dorsal wall of the latter. The lateral (right and left) walls are thin layers of the endoderm limiting the lateral expansions of the fore-gut.

The hind-gut is the hindmost portion of the archenteron descending to the posterior side of the yolk mass. The lining of the dorsal lip of the blastopore forms its dorsal wall, while that of the ventral lip makes its posterior wall.

The mid-gut is a narrow canal dorsal to the yolk mass, which connects the fore-gut with the hind-gut.

When the tail is somewhat elongated and the external gills begin to appear (stage 19), there are considerable changes in the three portions of the archenteron, which has become remarkably extended in the antero-posterior direction (Text-fig. 1C).

In the fore-gut region the pharynx is distinguishable from the rest, forming the visceral pouches on its lateral walls. The liver diverticulum and the remnant of the blastocoel become united to form a very deep pocket of the endoderm. The mid-gut is so narrowed that it is found only as a vertical slit in cross-section. The dorsal wall of the hind-gut extends posteriorly into the tail, forming the 'post-anal gut' under the notochord. The remnant of the blastopore, the 'neurenteric canal', is already obliterated and the anus is newly open.



TEXT-FIG. 1. (*See opposite.*)

At the stage of completion of the external gills (stage 21), each part of the digestive tract becomes fairly distinct. Text-fig. 1D is a diagrammatic illustration of the tract at this stage. The mouth opening is formed at the anterior extremity of the fore-gut. From the hind end of the floor of the pharynx develops a small saccular evagination which will give rise to the lungs and trachea. The portion of the fore-gut posterior to this evagination descends postero-ventrally as a tube of the endoderm which is referred to as the 'gastro-duodenal tube'. It consists of the rudiment of the oesophagus, stomach, and duodenum, but the boundary of each component is not distinguishable by external appearance. Histologically, the rudiment of the stomach is distinguished from that of the oesophagus by a difference in its glands and from that of the duodenum by the position of the opening of the bile-duct. The liver develops as a large evagination projecting antero-ventrally from the hind end of the gastro-duodenal tube. A small vesicle posterior to it is the precursor of the gall-bladder.

On the left side of the liver there is found a small evagination, the 'ventral pancreas'. In subsequent stages it is incorporated to form the pancreas with the 'dorsal pancreas', which arises from the part of the archenteric roof covering the boundary between the fore-gut and the mid-gut.

The cavity of the mid-gut is quite vestigial (Plate, fig. A), its anterior end descending ventrally to approach the remnant of the blastocoel. In the hind-gut region, there is no noticeable change other than the obliteration of the post-anal gut.

As the operculum develops and the external gills are reduced, the intestine begins to form the double coil characteristic of the frog tadpole (stage 25). Each component of the digestive system becomes quite distinct (Text-fig. 1E). In the fore-gut region the oesophagus, stomach, and the anterior part of the duodenum are distinguishable by their external appearance. The dorsal rudiment of the pancreas unites with the ventral. The bile-duct is found leading from the gall-bladder into the anterior end of the mid-gut.

The most remarkable changes take place in the mid-gut region. The archenteric cavity in this region is entirely obliterated (Plate, fig. B). The definitive cavity of the intestine is newly formed by a split in the solid mass of yolk cells

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TEXT-FIG. 1. Four stages of the development of the digestive organs from late neurula to stage-25 larva. Dotted areas in figs. A–D show the endoderm for the prospective digestive organs. A, longitudinal section of stage-14 neurula. B, frontal section of stage-14 neurula. C, longitudinal section of stage-19 larva. D, diagrammatic longitudinal section of stage-21 larva. A broken line shows the passage of the archenteric cavity of the mid-gut just before its closure. E, dorsal view of digestive organs from pharynx to anus in stage-25 larva. *an*, anus; *bc*, remnant of blastocoel; *bd*, ductus choleducus; *bl*, blastopore; *duo*, duodenum; *fg*, fore-gut; *fg.a*, *fg.d*, *fg.p*, *fg.r*, and *fg.v*, anterior, dorsal, posterior, right, and ventral wall of fore-gut; *gb*, gall-bladder; *gl*, glottis; *hg*, hind-gut; *hg.a*, *hg.d*, *hg.p*, and *hg.r*, anterior, dorsal, posterior, and right wall of hind-gut; *int*, small intestine; *ld*, liver diverticulum; *liv*, liver; *lu*, lung; *lu.l*, left lung; *mg*, mid-gut; *mo*, mouth; *oes*, oesophagus; *or*, oral evagination; *p*, pancreas; *pd*, dorsal pancreas; *ph*, pharynx; *pv*, ventral pancreas; *re*, rectum; *st*, stomach; *tg*, post-anal gut.

which begins at both ends of the mid-gut and effects a junction in the middle portion (Plate, fig. C).

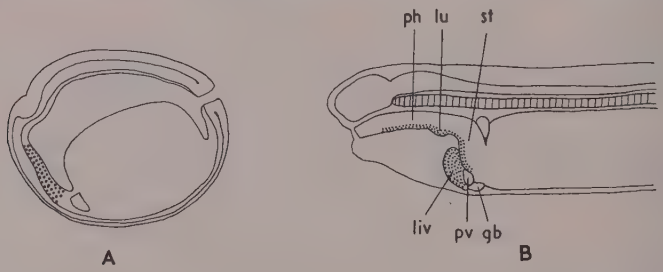
Histological observation of the newly opened intestine reveals that its wall is formed of one layer of columnar cells abounding in yolk granules. Held between the inner tips of these columnar cells there are also yolk cells, undifferentiated histologically and small in size. They may be identified as food cells, because similar cells are often observed degenerating in the intestinal cavity (Plate, figs. D, E). The hind-gut forms the rectal portion running antero-posteriorly across the dorsal side of the coiled intestine.

RESULTS OF VITAL STAINING

Staining of the fore-gut

Ventral and posterior walls

RF 541. The midline of the ventral wall of the fore-gut of a neurula at stage 14 was stained blue (Text-fig. 2A). Both ends of the wall were left unstained and the other walls were protected from the dye by cellophane. The specimen was dissected after the formation of the external gills (stage 20*a*) and the mark was found



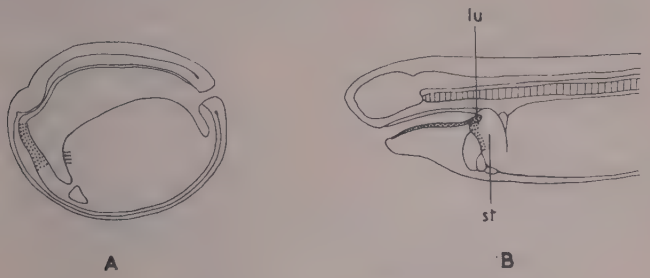
TEXT-FIG. 2. Staining of the ventral wall of the fore-gut (RF 541). A, area stained originally. B, location of the mark in stage 20*a* larva. The abbreviations are the same as those used in Text-fig. 1.

in the following areas (Text-fig. 2B): the ventral wall of the pharynx and oesophagus, the rudiments of the lung, the ventral wall of the gastro-duodenal tube, and the anterior wall of the growing liver. No sign of the dye was detected in the gall-bladder, in the ventral pancreas, or in the ventral portion of the liver.

RF 542. The original mark was placed as in RF 541, except that it reached to the posterior end of the ventral and lateral walls, i.e. into the liver diverticulum. In this case the gall-bladder, the ventral pancreas, and the ventral portion of the liver were also dyed.

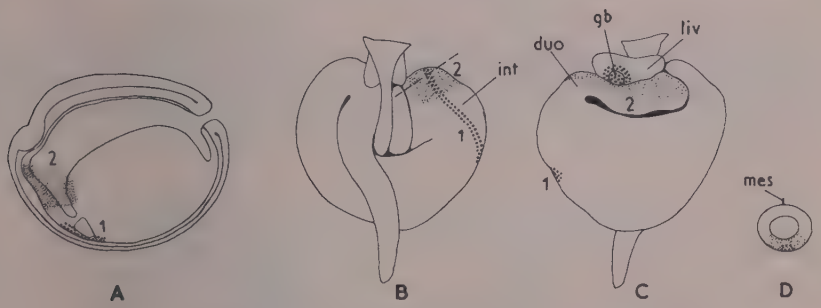
RF 543. The anterior half of the ventral wall was stained. The anterior end of the stained area coincided with that of the ventral wall, while the posterior end lay near the opening of the liver diverticulum (Text-fig. 3A). A small mark was also made on the posterior wall by transmission of the dye. In the final stage of external gill formation (stage 21), there was found a long band of dye extending

from the mouth to the end of the gastro-duodenal tube (Text-fig. 3B). The liver was quite free from stain. The mark on the posterior wall was traced to the ventral wall of the intestine (perhaps duodenum).



TEXT-FIG. 3. Another example of the staining of the ventral wall of the fore-gut (RF 543). A, area stained originally. B, location of the mark in stage-21 larva.

RFM 551. A piece of blue agar was applied, through an incision at the level of the remnant of the blastocoel, to the ventral surface of the endoderm (Text-fig. 4A). At the same time a red piece was inserted into the fore-gut through another incision in the oral region, to mark the greater part of the posterior wall and the



TEXT-FIG. 4. Staining of two portions of the ventral wall of the liver diverticulum (RFM 551). A, areas stained originally. B and C, location of the mark at an early stage of the spiral formation of the intestine. B is dorsal view and C is ventral view. D, cross-section of the duodenum on the same level with the broken line in B. 1, blue mark; 2, red mark; *mes*, mesentery.

anterior half of the ventral wall as well as the lateral walls connecting these two areas. At the beginning of spiral formation by the intestine (stage 23) a small portion of the blue mark lay in the gall-bladder and the posterior end of the liver, while the rest was found as a long band in the ventral wall of the duodenum and small intestine (Text-fig. 4 B, C, D).

From these results the following conclusions may be drawn:

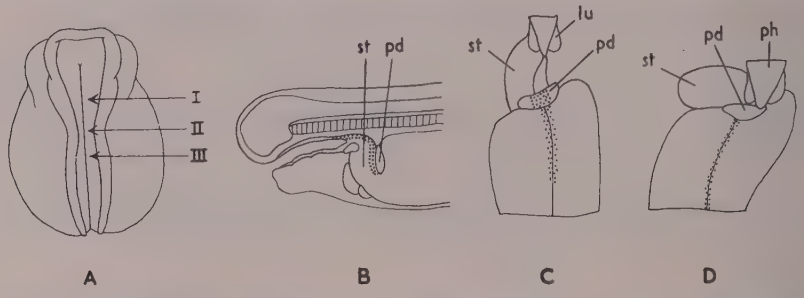
1. The anterior half of the ventral wall of the fore-gut forms the ventral wall of the digestive tract from the mouth to the anterior half of the duodenum; details of the fates of its various parts are illustrated in Text-fig. 22.

2. The posterior half of the ventral wall of the fore-gut, i.e. the floor of the liver diverticulum, develops for the most part into the liver, its hindmost part giving rise to the gall-bladder and the ventral pancreas.

3. The posterior wall of the fore-gut incorporates the posterior end of the ventral wall to form the ventral wall of the posterior half of the duodenum.

Dorsal wall

A series of operations was performed on neurulae at stage 14 to investigate the dorsal and anterior walls of the fore-gut. In each specimen, an incision was made through the neural plate and its substratum. The wound was plugged with



TEXT-FIG. 5. Staining of the dorsal wall of the fore-gut (RF 546–8). A, levels stained originally. Arrows indicate the portions in which the coloured agar was inserted. B, C, and D, situation of the mark in stage-21 larvae. B is left side view; C and D are dorsal views. The stomach is pulled out anteriorly in C.

TABLE

Results of staining of the dorsal wall of fore-gut

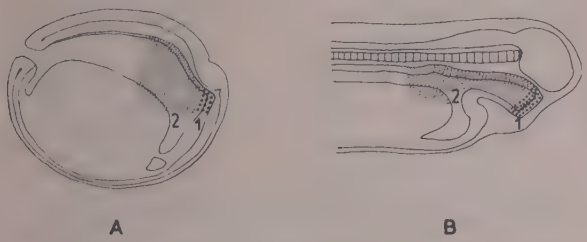
Specimens	*Text-figures*	*Levels marked originally*	*Final situation of the stain*			
			Pharynx	*Gastro-duodenal tube*	*Dorsal pancreas*	*Intestine*
RF 546	5B	I	+	+	−	−
RF 547	5C	II	−	−	+	+
RF 548	5D	III	−	−	−	+

a small piece of dyed agar, which was taken out after a few minutes. By this method the staining of the endoderm could be restricted to a small area. The operated levels varied according to the specimen. The following three (RF 546–8; Text-fig. 5A) are well worth describing: the anterior end (I), the middle (II), and the posterior end (III) of the narrowed portion of the plate. The final position of the marks in stage 21 are summarized in the Table and illustrated in Text-

fig. 5 B, C, D respectively. The stain was always found in the dorsal wall of the digestive tract and sometimes in the dorsal pancreas. The dorsal pancreas is derived from the endoderm just beneath the middle of the narrow portion of the plate, the gastro-duodenal tube originating from the area just anterior to it and the intestine from the region posterior to it (see Table).

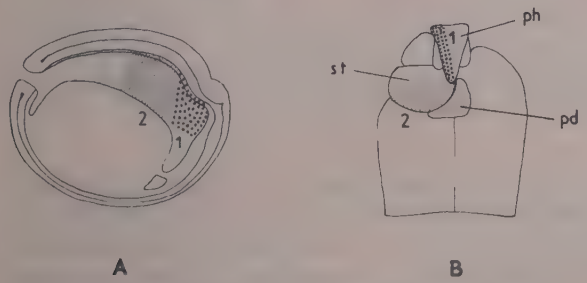
Anterior and lateral walls

RF 544. A blue mark (mark 1 in Text-fig. 6A) covered originally the endoderm of the oral evagination. Another mark, red and very large (mark 2 in Text-fig. 6A), was placed posteriorly to it, covering the whole region of the dorsal and left



TEXT-FIG. 6. Staining of the anterior and lateral wall of the fore-gut (RF 544). A, areas stained originally. B, location of the marks in stage-19 larva. 1, blue mark; 2, red mark.

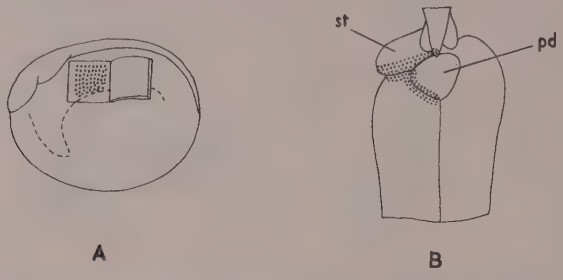
walls of the fore-gut and the anterior part of the mid-gut as well. The specimen was dissected in the early stage of external gill-formation (stage 19). The blue stain was found restricted to the endoderm of the oral plate. The red mark not only spread over the dorsal and left walls of the pharynx, but also extended to the walls of the mid-gut (Text-fig. 6B).



TEXT-FIG. 7. Another example of the staining of the anterior and lateral wall of the fore-gut (RF 545). A, areas stained originally. B, location of the marks viewed dorsally in stage-21 larva. 1, blue mark; 2, red mark.

RF 545. Two marks were arranged on the left wall of the fore-gut, the blue one anteriorly and the red one posteriorly (Text-fig. 7A). Both marks reached partly to the dorsal wall, so that the boundary between their dorsal portions

underlay the midbrain region of the neural plate. The specimen was reared until its external gills appeared (stage 21). The blue mark was traced to the left wall of the pharynx and the red one to the left wall of the gastro-duodenal tube (Text-fig. 7B). A portion of the latter was also detected in the dorsal pancreas. It was thus clearly shown that the boundary between the two marks coincides with the posterior end of the oesophagus.



TEXT-FIG. 8. Staining of the lateral wall of the fore-gut (RF 552). A, area stained originally. B, location of the mark viewed dorsally in stage-21 larva.

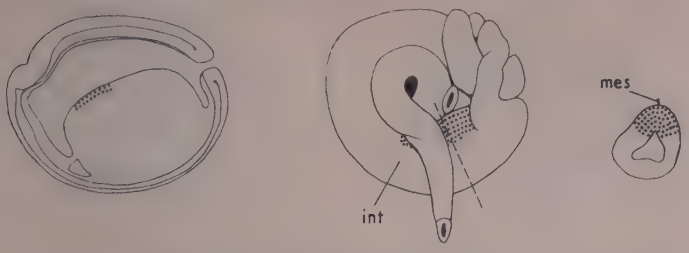
RF 552. As shown in Text-fig. 8A, the ectoderm and mesoderm on the lateral side of a neurula were incised in a ⌊-shape and the flap thus formed was lifted to expose the endoderm, against which dyed agar was pressed. By this method, the posterior end of the lateral wall of the fore-gut was stained from the outside. Afterwards, the stain was traced to the left side of the dorsal pancreas as well as to the lateral wall of the stomach and intestine (Text-fig. 8B).

It is seen from this result that the presumptive region of the dorsal pancreas is not restricted to the dorsal wall but extends to the lateral walls. Comparative study of the results described above leads us to the conclusion that the presumptive rudiments in the fore-gut region are located as illustrated in Text-fig. 22.

Staining of the mid-gut

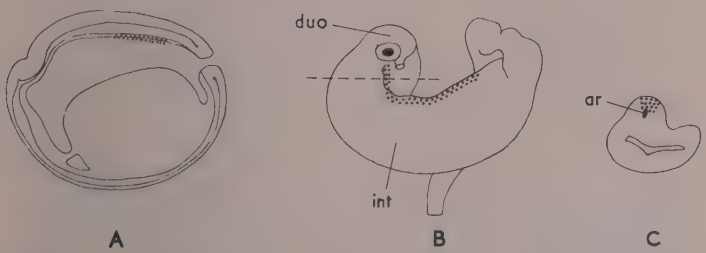
It is well known that the greater part of the mid-gut is involved in the formation of the small intestine. The most important problem is the fate of the walls of the mid-gut at the time of the new formation of the intestinal cavity following the closure of the archenteron. The following experiments were therefore performed.

RM 541 (Text-fig. 9). A mark was placed in the midline of the anterior part of the floor of the mid-gut. Transmission of the dye to the roof was avoided by the cellophane-cover method. After the development of the intestine (stage 24), the stain was found on the dorsal wall of the small intestine, and not on the ventral one. A part of the dye was also detected on the dorsal wall of the duodenum.



TEXT-FIG. 9. Staining of the floor of the mid-gut (RM 541). A, area stained originally. B, location of the mark viewed dorsally in stage-24 larva. C, cross-section of the intestine at the level of the broken line in B. *mes*, mesentery.

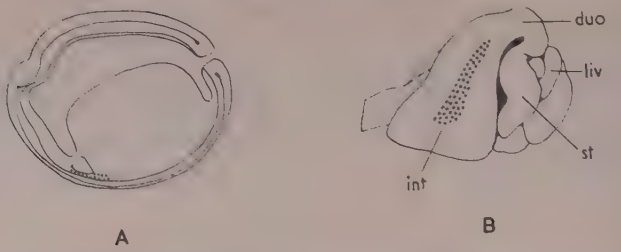
RM 542 (Text-fig. 10). A mark originally restricted to the roof of the mid-gut was traced on to the dorsal wall of the intestine and to the dorsal pancreas. In other examples, in which similar marking was done in the middle and caudal regions of the mid-gut, results were obtained in accord with those described above. All of the marks placed on the inside of the mid-gut were traced to the dorsal wall of the intestine.



TEXT-FIG. 10. Staining of the roof of the mid-gut (RM 542). A, area stained originally. B, location of the mark viewed ventrally in stage-23 larva. C, cross-section of the intestine on the same level with the broken line in B. *ar*, remnant of the archenteron of the mid-gut exceptionally present on the dorsal side of the true intestinal cavity.

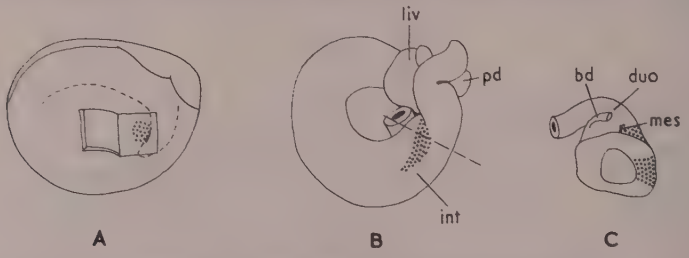
These facts demonstrate clearly that, in consequence of the closure of the mid-gut, its floor and lateral walls join with its roof to form the dorsal wall of the definite intestinal cavity, which is newly formed beneath the floor of the mid-gut, penetrating through the mass of yolk cells. Where then does the material for the lateral and ventral walls of the intestine originate? The answer will be found in the following specimens.

RM 551 (Text-fig. 11). The ventral ectoderm of a neurula was incised together with the underlying mesoderm. Through the wound, a piece of dyed agar was inserted to stain the antero-ventral surface of the yolk mass. After the formation of the intestine (stage 24), the stain was found stretched in a line on the surface of the most anterior portion of the ventral wall of the small intestine.



TEXT-FIG. 11. Staining of the ventral surface of the yolk mass (RM 551). A, area stained originally. B, right side view of the location of the mark in stage-24 larva.

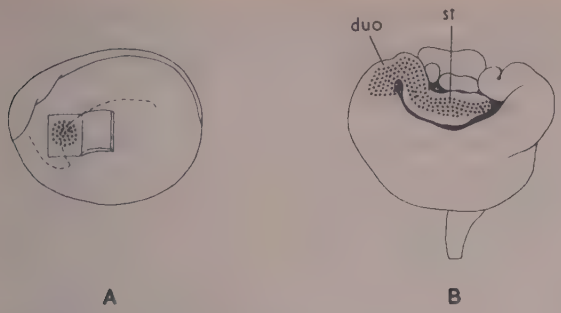
RM 552 (Text-fig. 12). Through a wound made in the ecto- and mesoderm on the right side of a neurula, a piece of coloured agar was applied to the right surface of the yolk mass. When the intestine was formed (stage 24), there was found a rather long and somewhat twisted stain on the right wall of the gut, extending from the duodenum to the anterior part of the small intestine.



TEXT-FIG. 12. Staining of the right surface of the yolk mass (RM 552). A, area stained originally. B, location of the mark viewed dorsally in stage-24 larva. C, cross-section of the duodenum at the level of the broken line in B. *mes*, mesentery.

RM 553 (Text-fig. 13). A mark somewhat larger in size was placed on the endoderm of the side opposite to that of the preceding case. At the time of dissection it was found on the left wall of the digestive tract as a long band running from the stomach to the small intestine. It is clearly shown by these examples that the lateral and ventral portions of the yolk mass contribute to the formation of the intestinal walls of the lateral and ventral sides respectively.

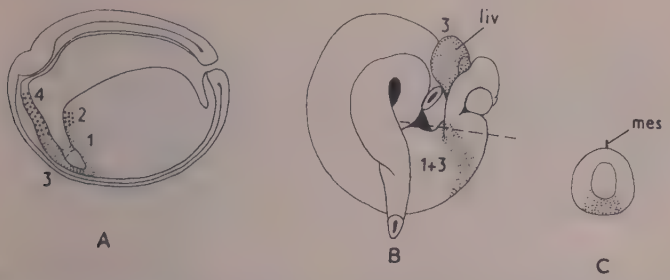
On the other hand, it has been mentioned (p. 144) that the material of the ventral wall of the duodenum derives also from the posterior wall of the foregut, i.e. the roof of the liver diverticulum. The epithelium of the duodenum therefore comes from two sources. The relation of these materials in the duodenal wall is illustrated in Text-fig. 4B. The stain originally placed on the roof of the liver diverticulum occupies almost the whole thickness of the wall but shows little extension, while the other, originally placed on the ventral surface of the yolk mass, stretches as a long line on the outer surface of the wall.



TEXT-FIG. 13. Staining of the left surface of the yolk mass (RM 553). A, area stained originally. B, location of the mark viewed ventrally in stage-23 larva.

Opening of the intestinal cavity into the fore-gut

As mentioned above, the archenteric cavity in the mid-gut is gradually closed up, a definitive cavity of the intestine being newly formed through the mass of yolk cells. Where is the opening of the latter into the fore-gut made? The following two examples suggest an answer to this question.

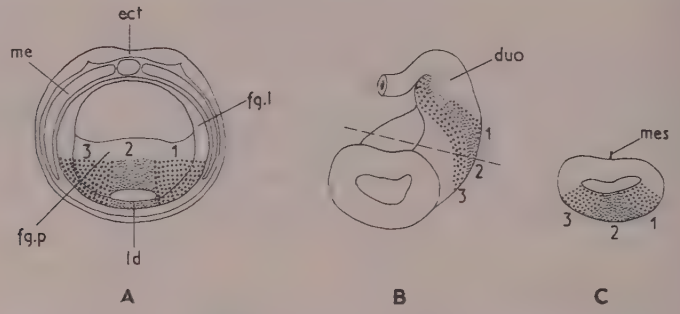


TEXT-FIG. 14. Staining of the walls of the liver diverticulum (RMF 554). A, areas stained originally. B, location of the marks in stage-24 larva. C, cross-section of the duodenum at the level of the broken line in B. 1 and 3, red marks; 2 and 4, blue marks; *mes*, mesentery.

RMF 554. A red piece of agar was inserted into the liver diverticulum by breaking through the septum between the latter and the rudimentary blastocoel. Another piece, blue in colour, was put anteriorly in close contact with it. Consequently, both dorsal and ventral walls of the inner part of the liver diverticulum were stained with red dye (marks 1 and 3 in Text-fig. 14A) while those near the entrance of the diverticulum were dyed blue (marks 2 and 4 in Text-fig. 14A).

The specimen was examined after the formation of the definitive intestinal cavity (stage 24). The red stain was detected in the liver, pancreas, gall-bladder, and also on the ventral wall of the small intestine; on the other hand, the blue dye was found only in the pharyngeal endoderm, and not in any portion of the

stomach or intestine (Text-fig. 14 B, C). From the data already presented it may be inferred that the blue stain originated from mark 4 in Text-fig. 14A, which was placed on the ventral wall of the fore-gut. Where then is mark 2, originally placed on the posterior wall? It was so dark that it can hardly be supposed to have faded out. It must have been obliterated as the result of the degeneration of the cells containing it at the time of perforation of the surface of the yolk mass. The area occupied by mark 2 may therefore be the site where the formation of the intestinal cavity begins. This conclusion is in quite good harmony with the fact that the mark on the anterior edge of the yolk mass was found on the *dorsal* wall of the anteriormost part of the small intestine (see Text-fig. 9B), whereas mark 1 in Text-fig. 14A was displaced to the *ventral* wall of the same part of the tract.



TEXT-FIG. 15. Staining of the walls of the liver diverticulum (RMF 555). A, semi-diagrammatic representation of the posterior half of the fore-gut of the neurula viewed anteriorly, showing the areas stained originally. B, part of the duodenum and the most anterior part of the small intestine cut off from stage-24 larva. C, cross-section of the duodenum at the level of the broken line in B. 1 and 3, blue marks; 2, red mark; *ect*, ectoderm; *fg.l*, lateral wall of fore-gut; *fg.p*, posterior wall of fore-gut; *me*, mesoderm; *mes*, mesentery.

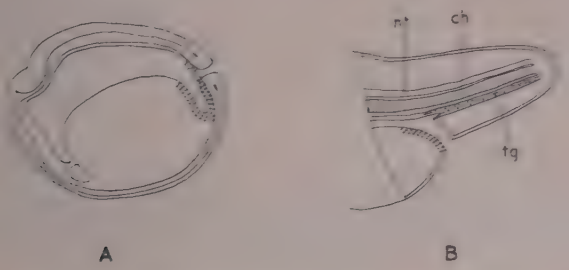
RMF 555. As illustrated in Text-fig. 15A, three marks—blue, red, and blue—were arranged laterally across the posterior wall of the fore-gut. Afterwards, all of them were traced on to the *ventral* wall of the entrance of the intestine, keeping their original arrangement (Text-fig. 15 B, C). Such a fact demonstrates that the aperture of the intestine is formed in the lateral direction across the dorsal half of the posterior wall of the fore-gut.

Staining of the hind-gut

The closure of the archenteric cavity does not take place in the hind-gut region. The prospective fate of each part of the walls of the hind-gut was studied as follows.

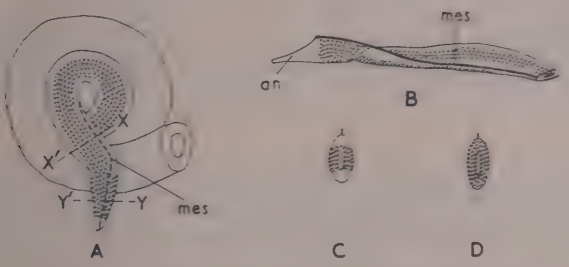
RH 551. A piece of dyed agar was inserted into the cavity of the hind-gut, so that the anterior, ventral, and posterior walls were stained in their median portion. The endoderm surrounding the blastopore was also stained

(Text-fig. 16A). At the appearance of the external gills (stage 19) the dye was found not only in the roof and floor of the hindmost part of the archenteron but also in the whole region of the post-anal gut (Text-fig. 16B).



TEXT-FIG. 16. Staining of the walls of the hind-gut (RH 551). A, area stained originally. B, longitudinal section of the posterior trunk and tail regions in stage-19 larva, showing the situation of the mark. *ch*, notochord; *nt*, neural tube.

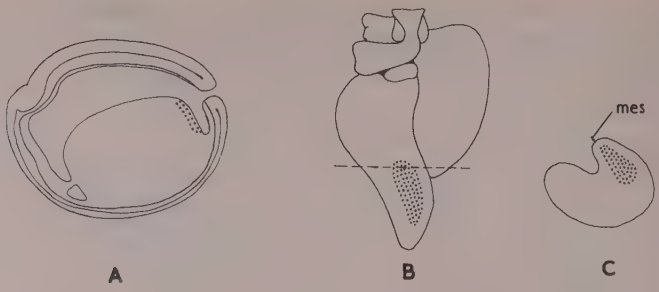
RH 552. The stain was applied as in the preceding example. The specimen was reared until the spiral formation of the intestine had proceeded to a considerable degree (stage 24). The dye was detected mostly on the lateral walls on both sides



TEXT-FIG. 17. Another example of the same staining as shown in Text-fig. 16 (RH 552). A, situation of the mark viewed dorsally in stage-24 larva. B, right side view of the rectum cut off from the same specimen, showing the situation of the mark. C and D, cross-section of the rectum at the level of the broken lines in *A*, *X–X'* and *Y–Y'*, respectively. *mes*, mesentery.

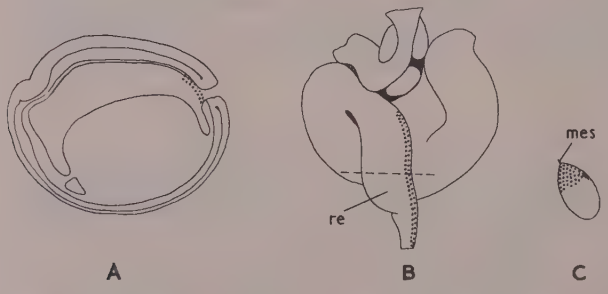
extending from the posterior part of the small intestine to the anus. The dorsal and ventral walls were found stained only in the end portion of the rectal tube (Text-fig. 17 A–D).

RH 541 (Text-fig. 18). By the method of cellophane-cover, the stain was restricted to the anterior wall of the hind-gut. In the stage of external gill appearance (stage 23), the mark was located within the yolk cells in the posterior portion of the intestine, which still did not have its definitive cavity.



TEXT-FIG. 18. Staining of the anterior wall of the hind-gut (RH 541). A, area stained originally. B, location of the mark in stage-23 larva. C, cross-section of the intestine at the level of the broken line in B. *mes*, mesentery.

RH 542 (Text-fig. 19). The dorsal wall alone of the hind-gut was stained. After the external gills were formed (stage 23–24) the stain was observed exclusively on the dorsal wall at the end of the intestine including the rectal region.



TEXT-FIG. 19. Staining of the dorsal wall of the hind-gut (RH 542). A, area stained originally. B, location of the mark in stage-23–24 larva. C, cross-section of the rectum at the level of the broken line in B.

The following conclusions may be drawn from these results:

1. The dorsal wall of the hind-gut, i.e. the endoderm lining the dorsal lip of the blastopore, forms the dorsal wall of the tract extending from the hind end of the small intestine to the rectum.

2. The anterior wall is split along its median line (in the dorso-ventral direction) by the opening of a definitive intestinal cavity into the hind-gut, so that its main portion is divided into right and left halves and involved in the formation of the hindmost part of the intestine.

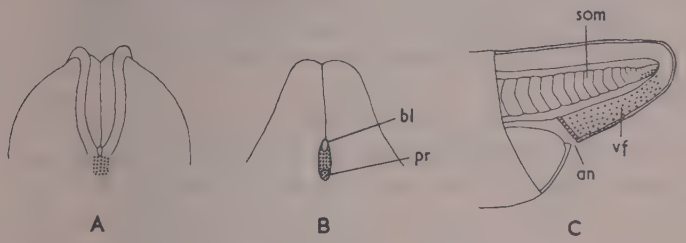
3. The ventral wall is incorporated into the formation of the ventral wall of the rectum.

4. The post-anal gut arises from the endoderm at the margin of the blastopore, for the most part from the endoderm lining the ventral lip.

Formation of the anal tube

There still remains a problem unaccounted for in the preceding description, namely, the formation of the anal tube. It is well known that in Anura the anus does not originate from the blastopore, but is newly formed by perforation of the posterior wall of the hind-gut. In the present study, the location of its rudiment in the neurula and the site where the perforation occurs were investigated by vital staining. In order to examine the final situation of the dye exactly, specimens were sectioned and observed microscopically.

RP 552. A rectangular mark was placed on the ventral lip of the blastopore of a neurula in the middle stage (Text-fig. 20A). Its length was twice that of the blastopore (in the dorso-ventral direction) and its width $1\frac{1}{2}$ times that of the blasto-



TEXT-FIG. 20. Staining of the ventral lip of the blastopore (RP 552). A, area stained originally. B, posterior view of the location of the mark in the embryo immediately after the closure of the neural folds. C, left side view of the location of the mark in the posterior trunk and tail regions of stage-19 larva, the epidermis having been stripped off. *pr*, proctodaeum; *som*, somite; *vf*, ventral fin.

pore (in the lateral direction). Its ventral edge was at nearly the same level as the ventral wall of the hind-gut. Just after the closure of the neural folds it was found in the groove formed on the ventral side of the blastopore (Text-fig. 20B). On the lower end of the mark there appeared a small pit which soon became perforated to give rise to the aperture of the anus. For a brief space of time the blastopore and the anal pit were observed side by side, keeping a short distance from each other. Soon afterwards, the former was covered by the epidermis to form the neurenteric canal, and the latter alone was left open. When the tail was somewhat elongated (stage 19) the dye was found on the ectodermal wall of the anal tube and in the somites of the tail end as well as in the mesenchyme cells and epidermis of the ventral fin (Text-fig. 20C).

RP 551. Dorsal and lateral lips of the blastopore were stained in the middle stage of the neurula (Text-fig. 21A). When the tail had grown somewhat (stage 19) a microscopical examination was made, revealing the dye in the somites and neural tube of the tail region and also at the caudal end of the notochord. No trace of the dye was found in the anal tube (Text-fig. 21B).

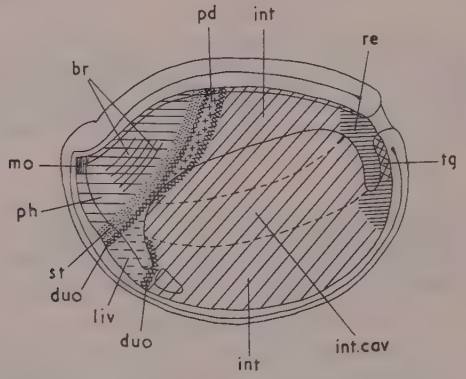
These results show clearly that the posterior wall of the hind-gut is perforated

through its ventral half to form the anus. The material for the proctodaeum is located on the ventral side of the blastopore and not on the dorsal or on the lateral side.

TEXT-FIG. 21. Staining of the dorsal and lateral lips of the blastopore (RP 551). A, area stained originally. B, left side view of the location of the mark in stage-19 larva, the epidermis having been stripped off in the posterior trunk and tail regions. *nt*, neural tube; *som*, somite.

CONCLUSIONS

The results of the present study have been synthesized into a map of the prospective digestive organs in the neurula, shown as Text-fig. 22.



TEXT-FIG. 22. A fate-map of the presumptive digestive organs in the neurula (stage 14) of *R. nigromaculata*. The position of each presumptive material is inscribed on the cut surface along the median line and on the right wall of the archenteron. *br*, branchial pouches (I–V); *duo*, duodenum; *int*, small intestine; *int.cav*, intestinal cavity; *liv*, liver; *mo*, mouth; *pd*, dorsal pancreas; *ph*, pharynx; *re*, rectum; *st*, stomach; *tg*, post-anal gut.

Pharyngeal endoderm. The greater part of the fore-gut is devoted to the formation of the pharynx. The oral evagination, lying just below the transverse fold, is actually the rudiment of the mouth, as its name signifies. The lateral walls on both sides give rise to the branchial pouches. It is probable that the lungs are

derived from the area of the floor ventral to the hindmost pair of the pouches. The oesophagus will arise from the boundary between both pharyngeal and gastric regions.

Gastric endoderm. The posterior end of the fore-gut is occupied by two presumptive rudiments, both having the shape of very large but extremely slender rings. The anterior one is that of the stomach, and the posterior one that of the anterior half of the duodenum. The uppermost part of the anterior ring which underlies the rhombencephalic region of the neural plate will form the dorsal wall of the stomach, while the lowest part which is located about the entrance of the liver diverticulum will form the ventral wall. In consequence, the intermediate parts on both sides of the fore-gut will become the lateral walls of the stomach.

Duodenal endoderm. The material for the duodenum is contained partly in the fore-gut and partly in the mid-gut region. The part in the fore-gut is the posterior ring mentioned above. It is at first involved in the formation of the gastro-duodenal loop and afterwards forms the anterior half of the duodenum which reaches from the end of the stomach to a level somewhat anterior to the aperture of the bile-duct. The other occupies the most anterior part of the mid-gut region, but does not coincide with the mid-gut itself as a portion of the archenteron. The mid-gut is closed up later, and the definitive cavity of the duodenum is newly perforated beneath the floor of it. Consequently, not only the roof of the mid-gut, but also the walls and even the floor are incorporated into the dorsal wall of the posterior half of the duodenum. The cells on the lateral surface of the yolk mass contribute to the formation of the lateral walls, and those of the roof of the liver diverticulum as well as those on the ventral side of the vestigial blastocoel are concerned with the formation of the ventral wall.

In the anuran tadpole the duodenum is not distinct either from the remainder of the intestine or from the pylorus of the stomach, and it is hardly possible to designate both ends of the duodenum with accuracy. In the present map the extent of the presumptive area of the duodenum is shown only approximately.

Hepatic endoderm. The presumptive region of the liver occupies the greater part of both lateral and ventral walls of the liver diverticulum, being bordered by the material for the duodenum. In the hindmost part of this region are situated, besides the material for the hepatic lobes, those for the ventral pancreas, gall-bladder, and bile-duct. The ventral pancreas originates from the left wall and the gall-bladder from the ventral wall. It is probable that the material for the bile-duct is closely connected with that for the gall-bladder, but the actual location of it has not yet been determined.

Pancreatic endoderm. The presumptive rudiment of the pancreas consists of two components, dorsal and ventral. The latter has been described above; the former is located on the dorsal and lateral walls of the archenteron in the region underlying the most anterior part of the spinal cord and forms the boundary between fore-gut and mid-gut.

Intestinal endoderm. The greater part of the endodermal cells in the mid-gut region, including those of the yolk mass, is concerned with the formation of the small intestine. As a consequence of the closure of the archenteron in this region and the new formation of the intestinal cavity, the floor of the mid-gut, incorporating the walls and roof, contributes to the formation of the dorsal wall of the intestine. The rest of the intestinal wall is derived from the other portions of the yolk mass; the lateral walls from the lateral portions and the ventral wall from the ventral portion, respectively.

The anterior opening of the intestinal cavity is formed as a horizontal slit in the dorsal half of the posterior wall of the fore-gut, just below the anterior edge of the yolk mass. On the other hand, the posterior aperture is perforated as a vertical slit along the median line of the anterior wall of the hind-gut. In the present map, the openings are connected by broken lines drawn across the yolk mass. These lines represent the prospective site of the definitive cavity of the intestine. However, neither the details of its course nor its width is known for certain. The only fact that is absolutely certain is that the intestinal cavity is newly formed through the yolk mass.

Rectal endoderm. The hind-gut is responsible for the formation of the rectum. Its roof develops into the dorsal wall of the latter, while its floor and subjacent yolk endoderm give rise to the ventral wall. The main portion of the anterior wall of the hind-gut, except for the median portion to be perforated, is involved, together with the endodermal walls on both sides, in the formation of the lateral wall of the rectum.

Post-anal endoderm. The endodermal rod in the tail, known as the 'post-anal gut', receives a contribution from the endoderm on the margin of the blastopore, for the most part from that lining the ventral lip. In the neurula stage the material for the post-anal gut is closely connected with that for the ventral wall of the rectum. These two materials are, however, separated later by the anus, which originates from the ectoderm of the ventral blastoporal lip.

DISCUSSION

Balinsky (1947) was the first investigator to apply the technique of local vital staining to the endoderm of the urodele neurula and to map the presumptive areas of the digestive organs. The most important of his findings is the closure of the mid-gut accompanied by the new formation of the intestinal cavity through the yolk mass. The present results show that the same is true in anuran embryos. Such a state of affairs seems to favour the old theory of Remak (1850) and to be out of harmony with the long-accepted idea of Goette (1875), who claimed the archenteric origin of the intestinal cavity. The discrepancy between these ancient authors may, however, be attributable to the different species adopted as their materials. In fact, we recorded in a previous paper (1954) an observation suggesting that the closure of the archenteron might be

much delayed in *Bombinator igneus*, the species used by Goette, with the result that the remainder of the mid-gut would incorporate the newly formed split in the yolk mass. In this way the new formation of the cavity is recognized as the general principle of the development of the amphibian intestine.

In spite of this agreement in principle, there are considerable differences between the present map for an anuran and Balinsky's for a urodele. In the latter, the liver diverticulum is designated as a rudiment of the digestive tract, including the stomach, the duodenum, and the foremost part of the small intestine. It goes on invaginating, according to Balinsky, deeper and deeper into the yolk mass till these primordia are shifted to their final situations. In the Anura, on the contrary, the same diverticulum is shown to be the material for the liver, as its name signifies. It was repeatedly substantiated in the present study that the greater part of the lateral and ventral walls of the diverticulum are devoted to the formation of the liver, the dorsal wall alone taking part in the formation of the duodenum. The presumptive rudiments of the stomach and of the anterior half of the duodenum were found in the fore-gut, occupying the hindmost part of the expanded wall, one-cell thick. These rudiments attain their destinations as a result of a drastic stretching and folding taking place in the anterior part of the archenteron in the course of subsequent development.

In order to throw more light on this apparent difference between Urodela and Anura, we have repeated the investigation on Japanese species of urodele. The results, to be published shortly, were very similar to those obtained in the present study on an anuran.

SUMMARY

1. The topography of the presumptive rudiments of the endodermal organs in the neurula of *R. nigromaculata nigromaculata* was studied by local vital staining. The results obtained are summarized in Text-fig. 22.

2. The main portion of the fore-gut develops into the pharynx, oesophagus, stomach, and the anterior half of the duodenum.

3. The lateral and ventral walls of the liver diverticulum give rise not only to the liver, but also to the gall-bladder, the bile-duct, and the ventral pancreas. The dorsal pancreas is derived from the dorsal and lateral archenteric walls at the boundary between fore-gut and mid-gut.

4. The yolk mass subjacent to the mid-gut is the material for the small intestine and the posterior half of the duodenum. The whole endoderm originally lining the mid-gut is incorporated into the dorsal wall in consequence of the closure of the archenteron and the perforation of the definitive cavity of the intestine through the yolk mass.

5. The hind-gut mostly forms the rectum; only a small portion at the margin of the blastopore becomes the post-anal gut.

RÉSUMÉ

Topographie des ébauches présomptives de l'endoderme chez la neurula des Anoures

1. La topographie des ébauches présomptives des organes endodermiques a été étudiée par des colorations vitales localisées chez *Rana nigromaculata nigromaculata*. Les résultats obtenus sont résumés dans la fig. 22 du texte.

2. La plus grande partie de l'enteron antérieur se développe en pharynx, œsophage, estomac et la moitié antérieure du duodénum.

3. Les parois latérales et ventrale du diverticule hépatique ne forment pas seulement le foie mais aussi la vésicule et les voies biliaires, ainsi que le pancréas ventral. Le pancréas dorsal provient des parois dorsale et latérales de l'archenteron mais à la limite entre l'enteron antérieur et moyen.

4. La masse vitelline sous-jacente à l'enteron moyen représente le matériel pour l'intestin grêle et la moitié caudale du duodénum. La totalité de l'endoderme revêtant primitivement l'enteron moyen est incorporée dans la paroi dorsale de ces mêmes segments du tube digestif en conséquence de l'oblitération de la cavité archentérique et de la perforation de la lumière intestinale définitive au travers de la masse vitelline.

5. L'enteron postérieur forme le rectum; seule une petite portion adjacente au blastopore en est dévolue au canal anal.

ACKNOWLEDGEMENT

We wish to express our cordial gratitude to Prof. C. H. Waddington who has kindly read this manuscript.

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EXPLANATION OF PLATE

Fig. A. Cross-section of the mid-gut region, showing the archenteron just before closure. ×160.

Fig. B. Cross-section of the mid-gut region of an embryo with its archenteron completely closed. ×120.

Fig. C. Frontal section of the intestinal region, showing the new cavity appearing as a split. ×130.

Fig. D. Cross-section of the small intestine just after the new formation of its cavity, showing the columnar epithelium of the intestine and the degenerating yolk cells. ×130.

Fig. E. Cross-section of the small intestine with its cavity well defined, showing the columnar epithelium of the intestine and the degenerating yolk cells. ×120.

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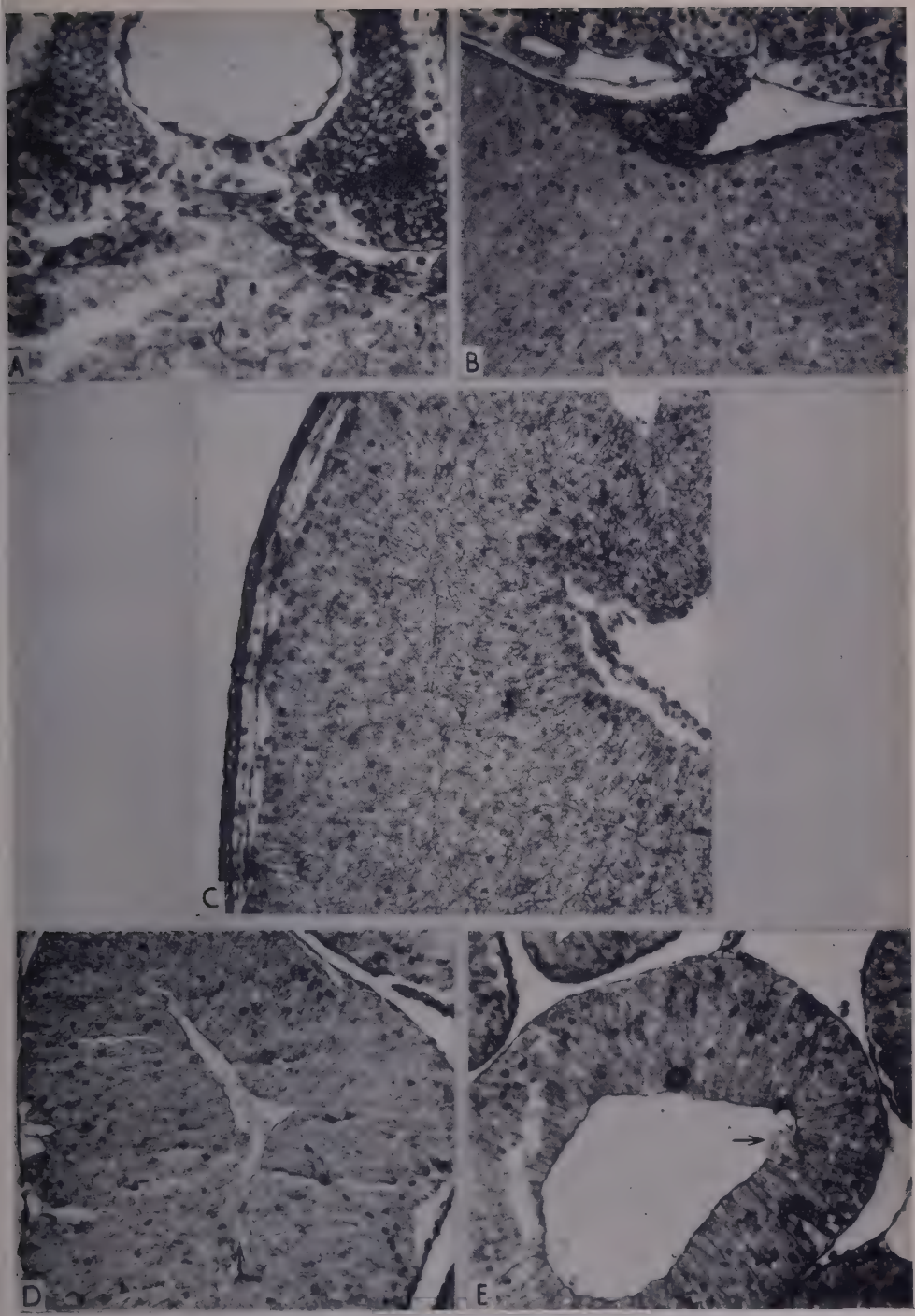
A
B
C
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Influence of Temperature on Rate of Regeneration in the Time-graded Regeneration Field in Planarians

by AGNES *and* H. V. BRØNDSTED[1]

From the Institute of General Zoology, University of Copenhagen

INTRODUCTION

THERE exists in planarians a time-graded regeneration field for head regeneration (Brøndsted, 1946, 1956; A. & H. V. Brøndsted, 1952). The characteristics of this field, expressed by rate of regeneration, are species-specific. The existence of this field ensures harmonious regeneration from cuts everywhere in the body, as a cut will always expose a 'high point' where regeneration of a head starts with greatest speed, thus taking the lead in organization and at the same time inhibiting head-forming tendencies elsewhere in the blastema (Brøndsted, 1956).

The factors underlying these characteristics of the field are unknown; the problems involved are being attacked from several angles in our laboratory. For the sake of this work it is of some interest to know how the different rates of regeneration at various levels in the time-graded fields might be influenced by various temperature levels.

MATERIAL AND METHODS

The experiments were carried out on two species differing greatly in the characteristics of their time-graded regeneration fields.

Euplanaria polychroa (Text-fig. 1) belongs to a group of species characterized by ability to regenerate a head from almost every part of the body, but at rates falling off evenly caudad and laterad (A. & H. V. Brøndsted, in preparation). *Bdellocephala punctata* (Text-fig. 2) has a time-graded regeneration field with a high point just behind the eyes; from here the rates fall off rather steeply to a level in the body just in front of the pharynx.

The temperature-gradient chamber of A. Krogh was used. It consists of a box approximately $100 \times 30 \times 30$ cm. made of galvanized sheet, insulated by soft plates made of compressed fibre-board. The box is divided into several compartments by watertight walls likewise made of galvanized sheet. When ice is placed in the first chamber and water in the rest, a temperature gradient is established ranging from about 1·6° C. in the chamber close to the ice chamber to about 16° C. in the farthermost chamber, when the room temperature is 20–22° C. The lid, likewise insulated, is pierced by holes through which thermometers are

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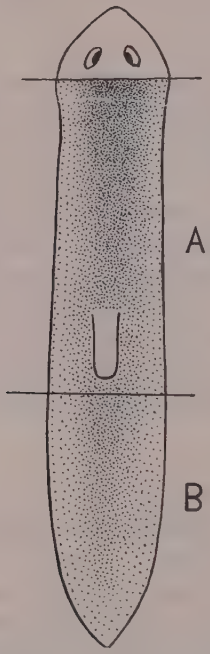
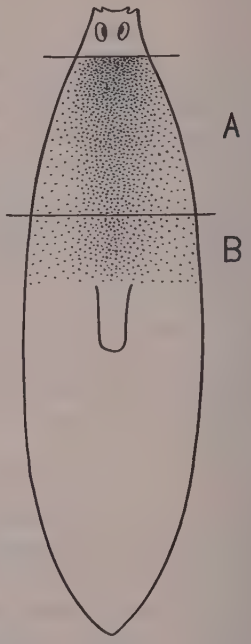
fitted dipping into the water of the chambers. In our experiments the ice was renewed every day at 8 a.m. and 8 p.m. The temperature stability in the compartments is fairly good, the variation not exceeding $\pm 1°$ C. The animals were placed in 200-ml. glass jars, floating in the water of the chambers. A control of a sort was established by putting animals in an incubator regulated to $20 \pm 0{\cdot}2°$ C. in general use for planarian regeneration in our laboratory.

Head-regeneration was regarded as fulfilled when eye-spots could be discerned in the binocular microscope at a magnification of 16 times with standard illumination.



TEXT-FIG. 1. Time-graded field of *E. polychroa*. The animals were cut in *A*- and *B*-pieces.



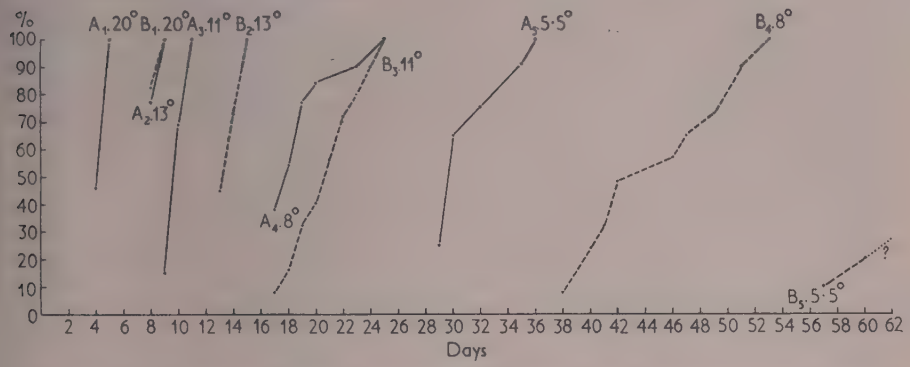
TEXT-FIG. 2. Time-graded field of *B. punctata*. The animals were cut in *A*- and *B*-pieces.

RESULTS

E. polychroa. Sixty-five specimens were cut as indicated in Text-fig. 1. Both the *A*-pieces and *B*-pieces were exposed to the following temperatures: A_1 and B_1 to $20 \pm 0{\cdot}2°$ C.; A_2 and B_2 to $13 \pm 1{\cdot}0°$ C.; A_3 and B_3 to $10{\cdot}9 \pm 0{\cdot}9°$ C.; A_4 and B_4 to $8{\cdot}3 \pm 0{\cdot}7°$ C.; and A_5 and B_5 to $5{\cdot}1 \pm 0{\cdot}5°$ C. The mortality was only slight—one or two specimens in a few batches. The duration of the experiment was 2 months. Text-fig. 3 gives the percentage of head-regeneration with time. The specimens were examined every morning.

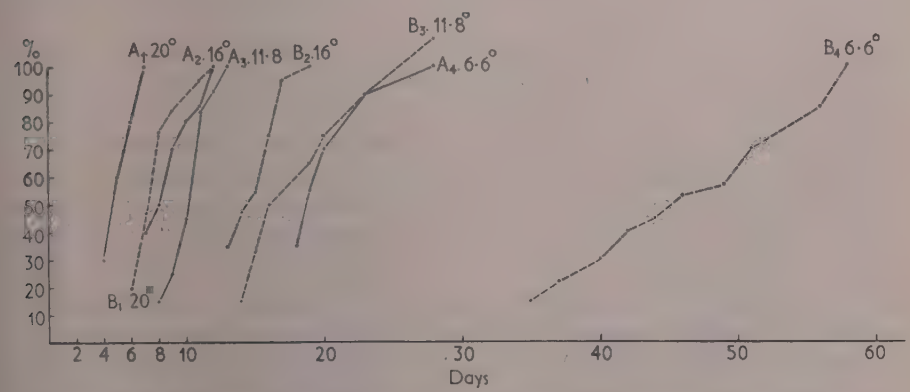
There are three salient features. (1) As was, of course, expected, lower

temperatures have a retarding effect on the regeneration rate. (2) In each batch the spread of regeneration rates is more pronounced at lower temperatures, and the more so in the 'weaker' *B*-pieces. (3) Lowering of the temperature has a comparatively greater retarding effect on regeneration rate in 'weaker' parts of the field. The relationship of these features will be discussed later.



TEXT-FIG. 3. *E. polychroa.* Rate of regeneration of *A*-pieces (full lines) and *B*-pieces (broken lines) at various temperatures.

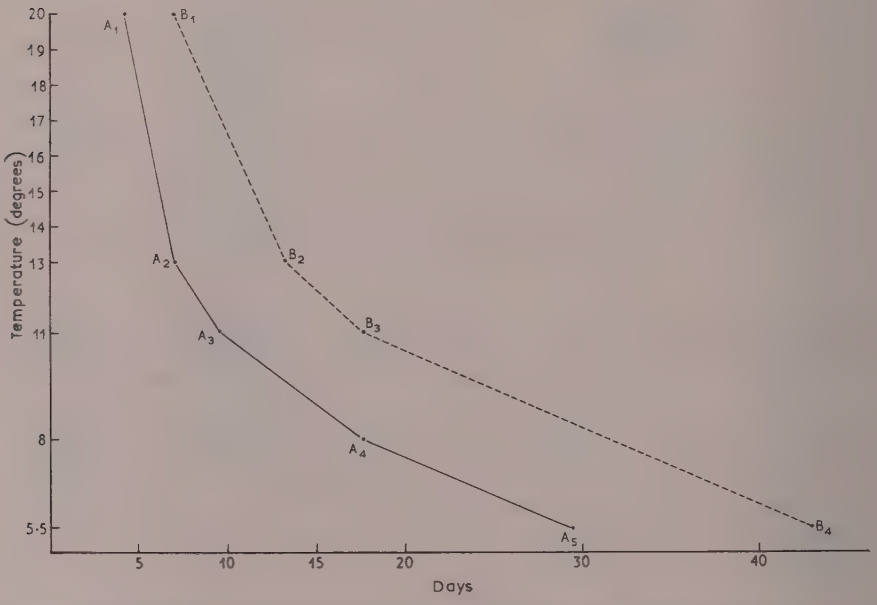
B. punctata. The animals were cut as indicated in Text-fig. 2, so that the time-graded field was cut at a high level of the field in the *A*-pieces and at a rather 'weak' level in the *B*-pieces. A hundred specimens were operated upon and were divided into five batches of 20 each. A_1 and B_1 were exposed to $20\pm0{\cdot}2°$ C.; A_2 and B_2 to $16\pm1{\cdot}0°$ C.; A_3 and B_3 to $11{\cdot}8\pm1{\cdot}0°$ C.; A_4 and B_4 to $6{\cdot}6\pm0{\cdot}6°$ C.; A_5 and B_5 to $1{\cdot}6\pm0{\cdot}4°$ C. Text-fig. 4 gives a survey of the results, which show the same three features already noted in *E. polychroa*. There was no regeneration of heads at a temperature of 1·6° C.



TEXT-FIG. 4. *B. punctata.* Rate of regeneration of *A*-pieces (full lines) and *B*-pieces (broken lines) at various temperatures.

DISCUSSION

One point must be stressed before evaluating the results. In cutting the animals at an intended level it is impossible to be exact, because even when slightly anaesthetized, the animals are contracted to a varying degree; the cuts will therefore be placed sometimes a little anterior to the intended level, sometimes a little posterior in the time-graded field; for this reason a slight spread of regeneration rate will occur in each batch. In addition, the different specimens undoubtedly show a slight variation in the rate of regeneration at a certain level. This spread has to be taken into account when interpreting the results. When the cuts are

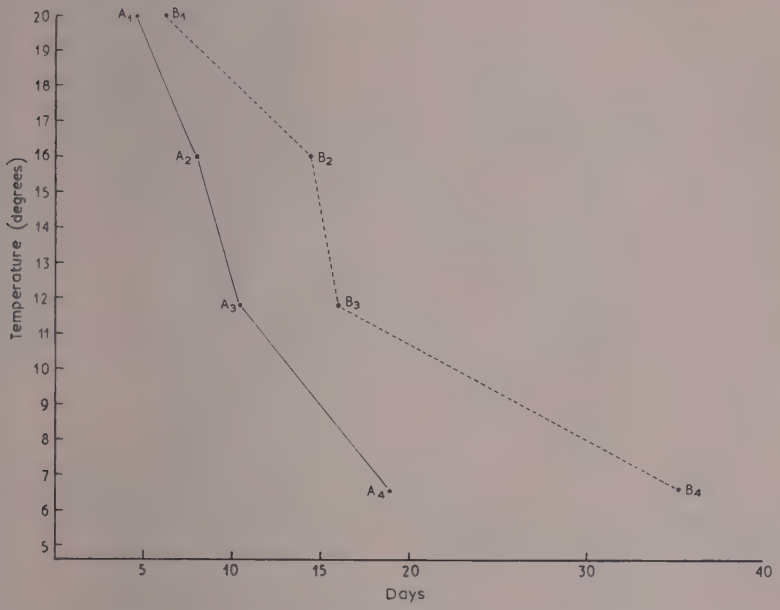


TEXT-FIG. 5. *E. polychroa*. Fifty per cent. regeneration of *A*-pieces (full line) and *B*-pieces (broken line) at various temperatures.

made just behind the eyes in *Bdellocephala* they strike a region where the field slopes only slightly, so that one would expect the spread to be rather small; whereas cuts in a region where the field is sloping more steeply must give a greater spread in a batch. The results of the experiments bear this out clearly, as seen in Text-figs. 3, 4, 5, and 6.

The first question to be considered is this: Are our findings consistent with the well-known gradient hypothesis of Child? Or do they confirm the findings of E. Løvtrup (1953) and Pedersen (1956), that no respiratory gradient exists, in either the normal or the regenerating animal? Child's hypothesis has been discussed in some detail by Brøndsted (1955).

It is, of course, easy to see that lowering the temperature will slow the regeneration processes, as these obey general physiological laws. But if a respiratory gradient existed in the animals, conforming to the time-graded regeneration field, then it seems to us that the regeneration rate would be depressed in the same way as the rate of a respiratory gradient, that is, the curves seen in Text-figs. 5 and 6 would run parallel. We therefore have to look for other explanations of the fact that the regeneration of the *B*-pieces is depressed more than that



TEXT-FIG. 6. *B. punctata*. Fifty per cent. regeneration of *A*-pieces (full line) and *B*-pieces (broken line) at various temperatures.

of the *A*-pieces. In this connexion it must be stressed that tail-regeneration and in fact all other regeneration than that of a head is very good everywhere in the body in both species. It is therefore not the regenerative power as such which follows the time-graded regeneration field of head-formation. It seems to us that the most plausible interpretation of our results is that special mechanisms are distributed throughout the planarian body in such a way as to be necessary and responsible for head-formation. This means that such mechanisms decline in amount posteriorly and therefore demand more time for expressing themselves in head-formation. Therefore the lower the temperature the greater the discrepancy in time which must ensue between regeneration from anterior and posterior parts of the body. Text-figs. 5 and 6 bear this out. These curves are drawn between points indicating 50 per cent. regeneration in each batch.

Table 1 gives the temperature coefficients for regeneration rates for intervals

of 10° C. throughout the entire range of temperature in which the experiments have been carried out. It is interesting to compare these coefficients for the two contrasting species. The exact validity of the coefficients must admittedly not be stressed too much, since the experimental conditions do not warrant this, but none the less we are confident that the experimental uncertainties are not

TABLE 1

Mean regeneration times of heads at two levels in the time-graded field at various temperatures

The coefficients are given for intervals of 10° C.

E. polychroa:	*A-pieces*		*B-pieces*	
Temperature	*Mean time of regeneration*	*Coefficient*	*Mean time of regeneration*	*Coefficient*
(° C.)	(days)		(days)	
20	4·2		7·0	
10	12·2	2·8	22·0	3·1
19	4·4		7·8	
9	15·0	3·4	23·6	3·0
18	4·8		8·6	
8	17·6	3·7	31·2	3·6
17	5·4		9·6	
7	22·0	4·1	36·0	3·8
16	5·8		10·5	
6	27·0	4·6	40·6	3·9
		Mean 3·6		Mean 3·5

B. punctata:	*A-pieces*		*B-pieces*	
20	4·6		6·2	
10	13·2	2·9	25·6	4·1
19	5·4		8·0	
9	14·8	2·7	31·4	3·9
18	6·2		10·1	
8	16·4	2·6	37·1	3·8
17	7·1		12·2	
7	17·9	2·5	52·8	4·3
		Mean 2·7		Mean 4·0

greater than the minimum required to bear out the general trends as seen in the figures. In the hands of experienced workers, batches of 10–15 specimens have been proved to be sufficient in experiments for working out the extension of the time-graded field in several species. In our experiments the two species behave differently in two respects. In *E. polychroa* (Table 1) there is a marked difference between the *A*- and *B*-pieces in so far as the *A*-pieces are influenced more strongly by lowering the temperature; such a difference is not found in *Bdellocephala*, where the coefficients are nearly the same at all temperature levels in

the *A*-pieces. In *E. polychroa* the mean of the coefficients at all temperature intervals is the same in *A*- and *B*-pieces, whereas in *Bdellocephala* there is a marked difference between the *A*- and *B*-pieces in this respect, the *B*-pieces being much more retarded than the *A*-pieces. It is, of course, impossible yet to interpret the results adequately; we are, however, inclined to believe that in *E. polychroa* the mechanisms responsible for head-formation are not so highly concentrated in the anterior region of the body as in *Bdellocephala*. Lender in various papers (1950–6) and Török (1958) have shown that the brain and other nerve-cells act as inductors for eye-formation. It seems rather probable therefore that the number of nerve-cells might be responsible for the rate of head-regeneration as detected by eye-formation. A clear-cut task would therefore be to investigate whether the contrasting power of head-regeneration in the two species used for our experiments depends on differences in the number of nerve-cells. Such an investigation is in progress in our laboratory.

However, the temperature experiments have shown another important feature. It was found that at lower temperatures the blastemata were formed a long time before the first eye-spots could be detected. This means that the wandering of the neoblasts to the wound was not seriously impeded at low temperatures. This is in accordance with the fact that both species at low temperature are very much alive in their natural surroundings in Lake Furesø near Copenhagen, where we have collected the animals. We have found newly laid cocoons at temperatures from 2 to 6° C. What is impeded by lowering the temperature is therefore the biochemical processes necessary for cellular differentiation.

At low temperatures, the strong retardation or complete stoppage of processes of differentiation necessary for fulfilment of regeneration makes the hypothesis of the adaptive value of regeneration doubtful; *Bdellocephala*, for example, has its egg-forming and egg-laying period from November to March, and the young are generally only hatched in April and May. So the *Bdellocephalas* have spent the better part of their lives at temperatures which impede regeneration.

SUMMARY

1. The aim of the experiments was to see how different levels in the time-graded head-regeneration field of two species, differing strongly as to the characteristics of the field, might behave at different temperatures.

2. As was expected, regeneration was retarded for all levels by lowering the temperature.

3. Regeneration of heads was retarded relatively more strongly at posterior levels of the field.

4. Some characteristic differences in regeneration rates of the two species were found, leading to certain suggestions as to the possible nature of the field.

5. By lowering the temperature regeneration may be separated into two main components: formation of the blastema and differentiation of the cells.

6. Formation of the blastema is not seriously impeded at temperatures below 5–6° C., whereas the differentiation processes are nearly brought to a stop at these low temperatures.

7. Doubt is therefore cast on the hypothesis of an adaptive value of regeneration in view of the fact that several planarian species actually live and reproduce at temperatures well below those necessary for regeneration.

ACKNOWLEDGEMENTS

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Number of Neoblasts in the Intact Body of *Euplanaria torva* and *Dendrocoelum lacteum*

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INTRODUCTION

THE regeneration blastema in planarians is constituted by the totipotent neoblasts which migrate to the wound (Dubois, 1949). The rate of regeneration, measured by appearance of eyes in the blastema, shows a characteristic variation throughout the planarian body, thus constituting a static, time-graded regeneration field (Brøndsted, 1946). We do not know the mechanism underlying this species-specific feature. Neither do we know why some species, e.g. *Dendrocoelum lacteum*, are able to regenerate a head only from the part of the body lying anteriorly to the pharynx, whereas other species, e.g. *Euplanaria torva*, can regenerate a head from almost every part of the body. A possible explanation might be the number of available neoblasts. This idea was formulated by Curtis & Schulze (1934). They claim that in *Procotyla fluviatilis*, a species closely related to *D. lacteum*, the inability to regenerate a head from parts behind the pharynx is due to scarcity of neoblasts, as compared with species with greater regeneration ability. This claim, however, is disputed by some authors, although supported in varying degree by others (for references, see Brøndsted, 1955). As far as we know, the actual number of neoblasts in the planarian body has never been reported. We therefore thought it necessary to get quantitative information about the problem, particularly as *Dendrocoelum* and other species (e.g. *Bdellocephala punctata*) with restricted head-regeneration ability are often able to regenerate tail and other parts quite easily from all parts of the body. We also think that a clarification of the problem has some significance for regeneration problems in general.

MATERIAL AND METHODS

D. lacteum and *E. torva* from Lake Furesø near Copenhagen were selected on account of their very differing power of head regeneration, as mentioned in the introduction. The animals were fixed in various fixatives; the best proved to be Zenker. Sections were stained with pyronin-methyl green by the method of Kurnick (1952, 1955), embedded in paraffin, and cut in transverse sections of 10 μ in order to transect as few neoblasts as possible without too great impairment

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of transparency. The neoblasts are rich in RNA (Pedersen, 1959), and in the sections they stand out clearly with an intense dark purple colour against other cell types, which may also be stained by pyronin. During microscopical observation the colour may be intensified by a dark yellow filter. The counting was done at a magnification of 100. To obtain as objective readings as possible, one of us counted the neoblasts in every section throughout the whole body of the animal, the other noted the dictated numbers without comment on any trend in the numbers. Several sections were examined twice at varying time intervals, and the counts of neoblasts never differed by more than 5 per cent. of the total.

We are, of course, quite aware of the error introduced by the risk of counting a transversely cut neoblast twice in two consecutive sections. The error was minimized by omitting a count if a neoblast seemed to be without a nucleus or had only a small portion of one; we therefore deemed it unnecessary to correct our findings by using the formulae given by various authors for proportionality of cell size and thickness of section. We have found no noteworthy difference in nuclear size of neoblasts in the two species, nor in other planarian species we have had occasion so far to investigate; the curves for the two species used here are therefore quite comparable as to the distribution of neoblasts measured with our method.

The volume of the fixed and embedded animals was measured by drawing the contour of every 5th section on squared paper and computing the areas of the sections; the figures found were plotted along the longitudinal axis of the animal (Figs. 1, 2).

RESULTS

D. lacteum. In a specimen, 15 mm. in the living and outstretched state, 37,826 neoblasts were found; they often lie in clusters, especially ventrally in the neighbourhood of the large lateral nerve-cords, but a statement of their more precise location is beyond the scope of this paper. Here we present only their distribution along the antero-posterior axis of the animal. Text-fig. 1 shows three curves: one representing the cross-sectional area of the animal along the main axis, in the fixed and embedded state; this curve is somewhat irregular on account of the unavoidable contractions taking place during fixation. The second curve represents the time-graded capability of head-regeneration, and the third curve gives the number of neoblasts. As will be seen, no neoblasts are found in the foremost part of the head region; from here the numbers rise suddenly, reaching a peak somewhat in front of the pharynx; the numbers fall in the pharyngeal region, rising again to a new peak around the genital apparatus, then falling abruptly to the most posterior part of the body.

Two points stand out: the distribution of the neoblasts coincides neither with the volume of the body nor with the capability of head regeneration.

E. torva. In a specimen, 15 mm. in the living and outstretched state, 31,320 neoblasts were found. Text-fig. 2 shows two distribution peaks placed in about

the same region as in *Dendrocoelum*. A slight difference may, however, be noted: the first peak is situated somewhat more anteriorly, and the caudal peak is slightly higher than the cranial. No correlation with cross-sectional area or time-graded field can be observed in this species either.

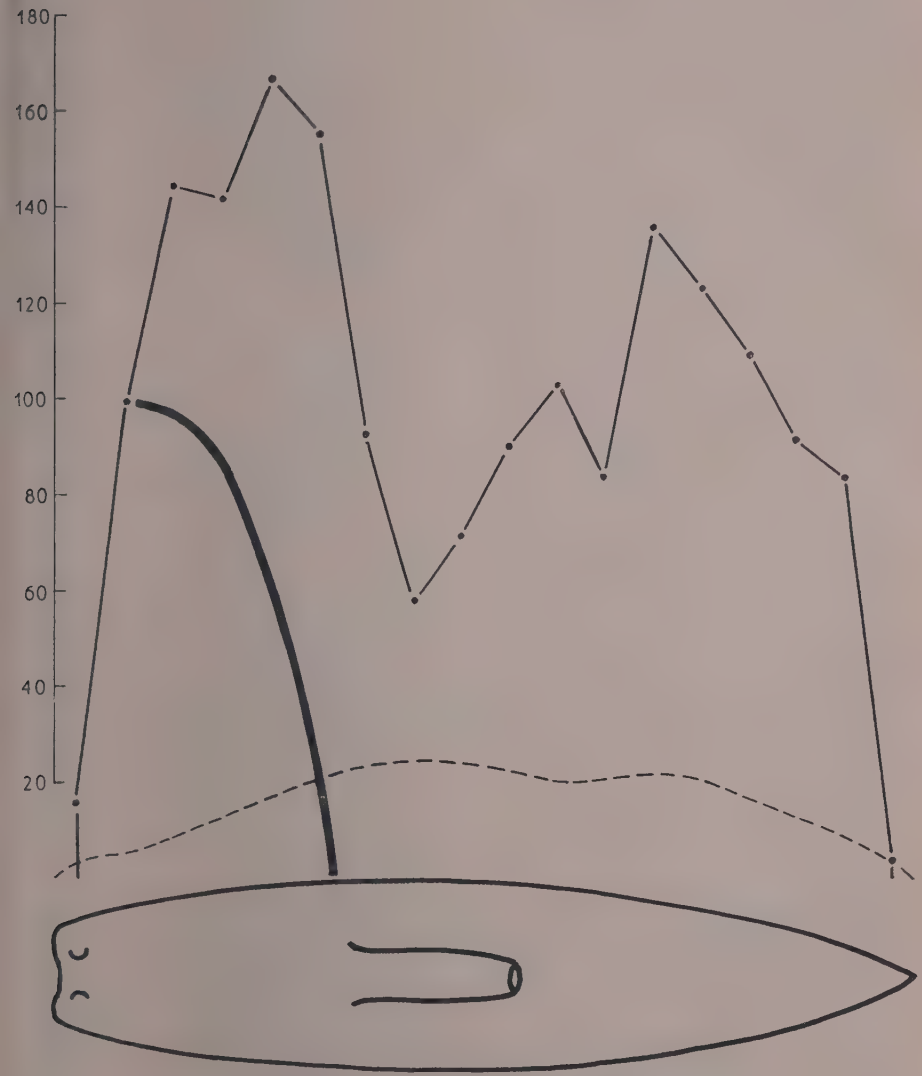

FIG. 1. *D. lacteum*. - - - - - cross-sectional area of the animal. —·—· numbers of neoblasts. — head-regeneration ability along the antero-posterior axis. The scale on the left indicates numbers of neoblasts.

FIG. 2. *E. lugubris.* - - - - - cross-sectional area of the animal. —·—· numbers of neoblasts. — head-regeneration ability along the antero-posterior axis. The scale on the left indicates numbers of neoblasts.

DISCUSSION

The hypothesis of Curtis & Schulze (1934), that the number of neoblasts might be decisive for the capacity to regenerate in species differing in this respect, has to be abandoned. It has been shown in this paper that the number and distribution of neoblasts do not furnish an adequate explanation why some planarian species are able to regenerate heads from every part of the body, although at different rates, whereas other species are able to do this only from the forepart of the body. A. & H. V. Brøndsted (1954) have shown that the rate of head regeneration is the same from small and large segments of the body, but that the size of the blastema formed depends on the neoblasts available (Brøndsted, 1956).

The species-specific character of the time-graded head-regeneration field must therefore be due to other factors, which are still completely obscure. Lender, in a series of papers (1950–6), has shown that the regenerating cephalic ganglia are responsible for eye regeneration. This fact is, of course, of great interest, but does not furnish a clue to understanding the underlying cause of the existence of the time-graded regeneration field. We are still ignorant on the question why the cephalic ganglia during regeneration originate at different rates at the various levels of the planarian body. We think that the fundamental problem of competence is involved, which has still to be unravelled.

Another question, probably quite separate from the one mentioned, is why the neoblasts are distributed in such a characteristic manner. This must involve delicately balanced biochemical processes, and is equally obscure. Both questions are being pursued in our laboratory.

It is interesting to compare our results in planarians with those of Tardent (1952–4) in *Hydra* and *Tubularia*. In these animals the RNA-packed neoblasts, designated I-cells by Tardent, are situated almost exclusively in the main body, not in the peristome and the tentacles; they are arranged in an antero-posterior gradient so that their numbers are greatest just behind the peristome. In Tardent's opinion, and he gives rather convincing reasons, this arrangement means that the neoblasts originate continuously at the high-point just behind the peristomal zone, and that they migrate continuously from here to places where physiological loss of cells has to be replaced by neoblasts by differentiation. Tardent has, however, not given any account of possible mitoses in the neoblasts in the zone of origin. No mitoses were seen in planarians.

The neoblasts in Hydroids are also responsible for true regeneration, and Tardent has shown that the regeneration ability follows the same gradient as the distribution of the neoblasts. When we compare these findings with those in the planarians, we see that other mechanisms must exist in the more highly organized planarians than those detected by Tardent in the much more lowly organized coelenterates.

It may be of some interest to note that the total volume of neoblasts in *E. torva* is about 0·0063 cu. mm., the average diameter of the neoblasts (in the fixed state) being about 6 μ (the cytoplasm being rather scanty). The total volume of the inspected specimen (in the fixed state) is 6·2 cu. mm., thus exceeding the volume of the neoblasts by a factor of 1,000.

Note added in proof: While this paper was in the press, two papers on the number of neoblasts in *Dugesia lugubris* appeared (Lender & Gabriel, 1960 *a*, *b*). They also reported two maxima, one just behind the eyes, the other just in front of the pharynx.

SUMMARY

1. The total numbers of neoblasts were counted in a specimen of *D. lacteum* and in a specimen of *E. torva*. The numbers were respectively 37,826 and 31,320.

2. The distribution of the neoblasts throughout the planarian body does not coincide either with head-regeneration ability or with cross-sectional area along the cephalo-caudal axis of these animals.

3. Although the distribution curve of the neoblasts shows fair agreement in the two species, the time-graded regeneration field is quite different, in *Dendrocoelum* stopping short just in front of the pharynx, in *Euplanaria* distributed throughout the animal (A. & H. V. Brøndsted, 1952).

4. The planarian time-graded regeneration field cannot therefore be ascribed

to the available numbers of neoblasts at the level of the cut, as is the case in hydroids.

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The Block to Polyspermy in Sturgeon and Trout with Special Reference to the Role of Cortical Granules (Alveoli)

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WITH THREE PLATES

INTRODUCTION

WHEN a monospermic egg is fertilized, the attachment of the fertilizing spermatozoon to the egg surface provokes a protective reaction that prevents all but one spermatozoon from entering the egg; this is the block to polyspermy. The nature of the defence mechanism against polyspermy has been the subject of many investigations, performed mainly on the eggs of sea urchins.

It was established that on fertilization of the sea-urchin egg a cortical reaction takes place consisting of morphological changes in the cortical layer, spreading in wave-like fashion from the point of the spermatozoon attachment over the whole egg surface: the light scattering and the intensity of birefringence undergo changes; extrusion of mucopolysaccharide granules takes place, accompanied by the separation of the vitelline membrane and its transformation into the fertilization membrane; the perivitelline space appears and the hyaline layer is then formed at the egg surface (see Runnström, 1952; Rothschild, 1956; Allen, 1958). Several workers have presented data suggesting that some of these changes take part in the inhibition of polyspermy.

However, the onset of the cortical reaction is preceded by a rather prolonged latent period, of the order of 10–20 seconds, while some 10–26 seconds more are necessary for the granule breakdown to sweep over the whole egg surface (Moser, 1939; Allen & Griffin, 1958). During such a time-lag many spermatozoa will collide with the egg. It was, therefore, suggested that the block to polyspermy can be ensured only by changes preceding the cortical reaction (Moser, 1939). Rothschild & Swann, 1952, Rothschild, 1956, brought forward the hypothesis that the process of block is diphasic: in the first phase invisible changes (the 'fertilization impulse') cover the egg surface within the course of a few seconds, leading to an incomplete block; the second phase corresponds to

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the visible cortical reaction and is completed with the formation of a sperm-impermeable layer (in *Psammechinus miliaris*, at a temperature of 16–18° C., in an average time of 63 seconds after insemination).

The purpose of this paper is to study the inhibition of polyspermy in fish and, in particular, to test on them the hypothesis of diphasic block. The mechanism of the block to polyspermy in fish has not been studied up to the present. Analysing the data found in the literature on the process of fertilization in fish, Rothschild (1958) suggested that the block to polyspermy was realized in them by means of the fertilization impulse.

The surface layer of teleost eggs is known to include cortical alveoli (cf. Rothschild, 1958), while that of Acipenseridae has cortical granules (Dettlaff, 1957, 1961); both of them contain mucopolysaccharides. As in the case of sea urchins, the contact of the fertilizing spermatozoon with the egg in fish initiates a wave of granule (or alveolus) breakdown. It seems interesting to elucidate whether this process plays some role in the block to polyspermy.

In order to find out the part played by the fertilization impulse and by the granule (alveolus) breakdown in the block to polyspermy, a series of experiments was done. (1) The course of the cortical reaction in normal development and after treatment with polyspermy-inducers was compared. (2) Sperm attachment and the onset of the cortical reaction were separated in time by activating the eggs and then inseminating them after various time-intervals; this experiment makes it possible to state the exact moment of the cortical reaction at which the penetration of the spermatozoon becomes impossible. (3) It was investigated whether the perivitelline fluid into which the content of the cortical alveoli is passed, inhibits the penetration of spermatozoa; with this aim in view (*a*) the perivitelline fluid was removed from activated eggs which then were inseminated, and (*b*) the direct action of the perivitelline fluid on spermatozoa was tested.

MATERIAL AND METHOD

The sturgeon *Acipenser güldenstädti colchicus* V. Marti and the lake trout *Salmo trutta* L. morpha *lacustris* Linné were the objects of investigation. Their eggs are covered by tough membranes, and the spermatozoa can reach the surface of the cytoplasm only through micropylar canals.

Sturgeon usually possess about 10 micropylar canals forming a group in the region of animal pole. In eggs taken from different females their number varies from 1–2 to 43. Under normal conditions of insemination only one spermatozoon enters the egg as a rule; while on insemination with fairly dense sperm suspensions, polyspermy is frequently observed (Ginsburg, 1957*a*, 1959). Simultaneous presence of several unchanged spermatozoa in fertilized eggs of *A. ruthenus* and *A. güldenstädti* was described by Perssov (1954, 1956, 1957). On these grounds he concluded that physiological polyspermy was natural in Acipenseridae. The penetration of supernumerary spermatozoa into the eggs of sturgeon, however, provokes far-reaching disturbances in development and

early death of the embryo (Ginsburg, 1953, 1957*a*) which is characteristic of physiologically monospermic eggs.

The egg of the trout has one micropyle, and specially carried out experiments showed that polyspermy is never observed, whatever the density of the sperm suspension at insemination. Thus the second object of investigation is also physiologically monospermic.

Experiments on sturgeon were carried out at the Rogozhkino sturgeon hatchery (the Lower Don) in the spring of 1958 and 1959. Eggs and sperm were procured from fish matured as a result of an injection with a homogenate of acetonized sturgeon pituitaries. Experiments on trout were performed at the Svir hatchery (Leningrad region) in October–November 1959. The fish were kept in river stews up to maturity.

The procedure of individual experiments will be dealt with in the corresponding sections. In the experiments that required an estimation of polyspermy incidence, the eggs with the characteristic cleavage pattern were counted. Hagström & Allen (1956) pointed out, in experiments involving nicotine treatment of sea-urchin eggs, that abnormal cleavage could not be used as a criterion of polyspermy. In the experiments on Acipenseridae, however, polyspermic eggs were always readily distinguishable from monospermic ones (even from those cleaving atypically) by the presence of superfluous blastomeres. The incidence of polyspermic eggs is completely revealed only at the stage of the second cleavage division, when counting was carried out. Special experiments showed that the percentage of eggs with superfluous blastomeres at this stage practically coincides with that of polyspermic eggs found in the same aliquot of eggs by means of cytological investigation (Ginsburg, 1957*a* and recent unpublished data). It is permissible therefore to regard the criterion chosen as a reliable one.

For cytological investigation the eggs were fixed with Sanfelice fluid and embedded in paraffin. In sturgeon only the animal part of the egg was as a rule embedded; the vegetative part was taken only for special purposes, since, because of its large amount of yolk, it is difficult to obtain satisfactory sections. In trout the cortical cytoplasmic layer or, later, the blastodisc was removed from the surface of the yolk. Serial sections of 7 μ, less often of 10 μ, were prepared. They were stained with Heidenhain's azan stain (cortical granules and the colloidal content of cortical alveoli are electively stained with the aniline blue) and with Heidenhain's iron haematoxylin. Drops and smears of trout-sperm were fixed with the vapour from 1 per cent. osmium tetroxide solution and stained with iron haematoxylin.

EXPERIMENTAL RESULTS

1. *Changes in the duration of the latent period and of the spread of the cortical reaction on pretreatment with polyspermy inducers*

The first attempt to approach the analysis of the block to polyspermy in fish consisted in a comparison of changes occurring in the sturgeon egg at

normal monospermic fertilization with those at experimental polyspermic fertilization induced by the action of chemical agents. It was shown on sea-urchin eggs (Rothschild & Swann, 1950; Rothschild, 1953; Hagström & Allen, 1956) that one such agent, namely nicotine, acts through the egg, prolonging the conduction time of the block to polyspermy (and not through increase of sperm motility). According to some results (Rothschild & Swann, 1950) nicotine does not affect the visible changes in the cortical layer, but according to others (Hagström & Allen, 1956), it inhibits the breakdown of the cortical granules and the formation of the hyaline layer. A retardation of the cortical reaction in sea-urchin eggs was also found on treatment with urethane, another polyspermy-inducing agent (Sugiyama, 1956).

In preliminary experiments on polyspermy induction in Acipenseridae under the action of urethane we were also able to confirm that its action was realized not through the spermatozoa but through the eggs. When the sperm was diluted in urethane solution, the percentage of polyspermic eggs either did not differ from that in the control or was less, but when eggs were treated prior to fertilization with the same solution, the polyspermy percentage constantly increased (Table 1).

TABLE 1

Incidence of polyspermy after treatment of sturgeon eggs and spermatozoa with 2 per cent. urethane

(Sperm density, $5{\cdot}55 \times 10^7$/ml.; temperature, 19·7° C.)

	Polyspermic eggs
	(%)
Eggs treated	17·3
Spermatozoa treated	0·5
Control (without treatment)	0·6

With the purpose of studying the mechanism of the action of polyspermy inducers, urethane, ethyl ether, and acetone were used. Ripe sturgeon eggs were pretreated for 5 minutes with a 2 per cent. urethane solution in river water. In two other variants of the experiments they were exposed to the action of ethyl ether or acetone vapours. To do this the eggs were distributed in one layer on gauze stretched over a glass ring; the ring was then immersed for 1 minute in a dish containing ether or acetone in such a manner that the layer of eggs was 10–12 mm. from the surface of the fluid. Immediately after the treatment the eggs were inseminated and aliquots of them were fixed at various time-intervals (from 10 seconds to 2 minutes) for cytological investigation (203 eggs were studied in sections). The remaining eggs developed up to the stage of the second cleavage division; the count of polyspermic eggs showed all the treatments applied to be effective (Table 2).

TABLE 2

Incidence of polyspermy in sturgeon eggs pretreated with urethane, acetone, and ethyl ether

(Sperm density, $7{\cdot}04 \times 10^7$/ml.; temperature, 21·2° C.)

Pretreatment	*Polyspermic eggs* (%)
2 per cent. urethane solution, 5 minutes	46·2
Acetone vapour, 1 minute	30·3
Ethyl ether vapour, 1 minute	26·9
Control (without pretreatment)	12·4

In an unfertilized egg the surface cytoplasmic layer that contains the cortical granules closely adheres to the egg membrane (Plate 1, fig. A). Ten seconds after insemination it can be seen that the cortical layer in almost all the animal region of the egg is strongly vacuolated, as a result of which the granules acquire a columnar form; the extrusion of the granules starts at some points, with the concomitant separation of the membrane from the egg surface (Plate 1, fig. B). In some eggs this process, as in the case illustrated, occurs only in small surface areas near the micropyles, while in other eggs it involves the whole top of the animal region. In the pretreated eggs at this time there have not yet appeared any indications of cortical reaction (Plate 1, fig. C). In acetone-pretreated eggs single large vacuoles (Plate 1, fig. D, *SV*), the result of the treatment applied, occur here and there at the egg surface.

Twenty seconds after insemination of the control eggs the granules are extruded and the membrane is separated over a large area (Plate 1, fig. E). In urethane- and ether-pretreated eggs the expulsion of the granules has also started, but in the first case it has spread only over a small distance (Plate 1, fig. F), while in the second case this process is just beginning (Plate 1, fig. G). After acetone pretreatment, the cortical layer is strongly vacuolated in the micropylar region, but the secretion of granules has not yet started (Plate 1, fig. H).

After 30 seconds the membranes in the control eggs are elevated in the whole animal region, but after urethane and ether pretreatment only in the centre of this region, while after acetone pretreatment granule breakdown has just started.

After 1 minute the elevation of the membranes in most of the control eggs is completed (in 6 out of 9, or 67 per cent.), while in the remainder the membranes have failed to separate from the surface of the egg only in a small region at the vegetative pole. In urethane-pretreated eggs the membrane elevation is at this time finished in a smaller percentage of cases (3 eggs out of 8, or 38 per cent.); after ether and acetone treatment the membranes of all eggs are not elevated at a considerable distance around the vegetative pole. After 2 minutes the

elevation of membranes is completed in all the experimental variants (with rare exceptions in ether- and acetone-pretreated eggs).

Thus all the treatments tested in this work that induced an increase in the incidence of polyspermy prolonged the latent period of the cortical reaction and apparently somewhat retarded the cortical granule secretion itself. The differences from the control are most clearly manifested early (10–30 seconds) after insemination, while later they become obscured. Most labile is the inhibiting action of urethane which easily penetrates into cells and can be rapidly released from them (Cornman, 1954). Since in sturgeon the establishment of contact between the spermatozoa and the eggs occurs in the first 10–15 seconds after insemination (Ginsburg, 1957*b*), it is just at this time that the inhibition of the block to polyspermy is of importance for the penetration of supernumerary spermatozoa.

The data of this set of experiments favour the existence of a direct correlation between the spread of the cortical reaction and the block to polyspermy. However, they do not provide indisputable evidence, since the chemical agents applied could have affected not only the cortical reaction but also the rapidly proceeding changes in the first phase of the block to polyspermy that have been suggested in the hypothesis of Rothschild & Swann.

2. *Loss of fertilizability after artificial activation of the egg*

(*a*) *Activation of sturgeon eggs by pricking*

In order to obtain more definite data on the relationship between the spread of the cortical reaction and the block to polyspermy, experiments were carried out in which the penetration of the spermatozoon and the onset of the cortical reaction were artificially separated in space and time. The results obtained have been published elsewhere (Ginsburg, 1960).

In these experiments the method applied was that used by Dettlaff (1961) when studying the role of fertilization impulse and cortical reaction in egg activation in Acipenseridae. Dettlaff pricked eggs at various points on their surface and thus varied the difference between the time taken by the fertilization impulse to reach the site of the nucleus and the time taken by the slower wave of cortical granule breakdown to reach the same place. Her experiments showed that the cortical reaction induced by pricking spreads at the same velocity as in fertilization.

In the experiments described below the sturgeon eggs were activated by pricking (*a*) near the animal pole (therefore near the micropylar canals), and (*b*) at the vegetative pole (at the maximum distance from the canals), and then inseminated. If changes blocking the entrance of spermatozoa spread over the egg surface in the first seconds after the activating treatment, the fertilizability of eggs in both experimental variants will be lost (or, at any rate, sharply decreased) almost simultaneously; if the inhibition is connected only with the visible cortical reaction, then in variant (*b*) the capacity to be fertilized will be

lost much later than in variant (*a*), the difference corresponding to the time required for the spread of cortical reaction over the egg (Dettlaff, 1957, 1961).

Sturgeon eggs were put into water one by one, pricked with a glass needle (diameter 20–40 μ), and after various time-intervals, from 1–2 seconds to 5 minutes, inseminated with rather dilute sperm (dilution 1:500 to 1:1000; sperm density, $1{\cdot}18\times10^6$ to $1{\cdot}21\times10^7$/ml.). In some lots the eggs stood such a treatment well, in others cytolysis occurred in many eggs. After pricking, all the eggs showed signs of activation. The difference between fertilized and activated but unfertilized eggs was manifested during cleavage (in activated eggs cleavage furrows do not appear at all or show an irregular pattern and appear with a considerable delay, Dettlaff & Ginsburg, 1954) and later, when fertilized eggs undergo gastrulation while activated ones gradually degenerate.

TABLE 3

Experiment (No. 3) on pricking animal and vegetative regions of sturgeon eggs with subsequent insemination

(Temperature, 13·8° C.)

Time from pricking to insemination	*Pricking the animal region*			*Pricking the vegetative region*		
	Total number of eggs	*Fertilized eggs*		*Total number of eggs*	*Fertilized eggs*	
			(%)			(%)
2 seconds	60	2	3·3	..	..	..
5 seconds	40	4	10·0	..	..	..
10 seconds	20	0	0	..	..	..
1 minute	..	..	..	20	16	80·0
2 minutes	..	..	..	20	19	95·0
3 minutes	..	..	..	30	13	43·3
4 minutes	..	..	..	20	0	0
5 minutes	..	..	..	20	0	0

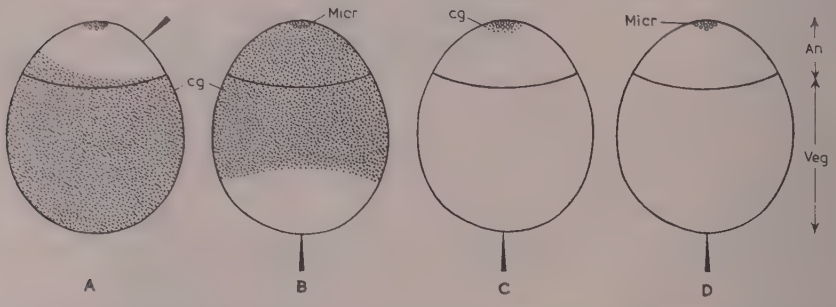
In all, 6 sets of experiments were performed and more than 1,000 eggs pricked, but only in 576 of them was development followed for a sufficient period of time. For cytological investigation eggs were fixed after the same time-intervals as when insemination was carried out. Seventy-four eggs were studied in sections.

All the experiments gave similar results. One of them will be taken as an example (Table 3). On pricking the animal region, the eggs were, as a rule, unfertilizable with the shortest intervals between pricking and insemination. Single eggs, however (an average of 6 per cent. in all experiments) became fertilized.

One can see in sections of the eggs that 5 seconds after pricking the wave of cortical reaction, spreading asymmetrically, reaches the border of the vegetative region at the side of the prick, while in the opposite direction it involves the whole top of the animal region (Text-fig. 1 A). At the position of the micropylar canals the escape of cortical granules is finished and the membrane is separated

from the egg surface (Plate 2, fig. A). It is evident that no spermatozoon entry into such eggs is possible.

Why occasional eggs become fertilized becomes clear as a result of living observations made after pricking. In many lots of eggs, in particular in darker pigmented ones, the wave of granule breakdown can be clearly seen: pricking is followed by a short latent period (usually lasting 1–2 seconds), a bright spot then appears at the site of pricking and spreads in all directions, towards the animal pole much faster than towards the border with the vegetative region. This wave reached the animal pole 3–5 seconds after pricking (at a temperature of 21–22° C.). It should be noted that some seconds must usually pass after insemination until spermatozoa cover the distance to the aperture of the micropyle and along the micropylar canal to the egg cortex (Ginsburg, 1957*b*). Even when insemination is done as soon as possible after pricking, the spermatozoon reaches the surface of the cytoplasm only when the cortical reaction has already spread over the micropylar region.



TEXT-FIG. 1. Scheme of the spread of cortical reaction in a sturgeon egg at various time-intervals after pricking the animal and vegetative regions (experiment No. 3, temp. 13·8° C.); A, 5 seconds after pricking the animal region; B, 2 minutes; C, 3 minutes; and D, 4 minutes after pricking the vegetative region. *An*, animal region; *cg*, cortical granules; *Micr.*, micropyle; *Veg*, vegetative region. That portion of the egg surface where cortical granules are discharged is white.

In occasional eggs, however, the cortical reaction proceeded more slowly, mainly at the expense of a prolongation of the latent period (similar differences in the duration of the latent period of cortical reaction were found in sea-urchin eggs by Allen & Griffin, 1958). In these eggs the wave of granule discharge reached the animal pole in 7–11 seconds. In such cases the spermatozoon can penetrate the egg when insemination occurred 2–5 seconds after the pricking.

On pricking the vegetative region (Text-fig. 1 B–D) fertilization occurred not only when insemination was a short time after pricking (5–30 seconds), but also after intervals of 1–3 minutes (Table 3). With intervals of 1 and 2 minutes, the fertilization percentage was high (87·5 per cent. on average) and practically corresponded to the fertilization frequency in the same lot of eggs inseminated

without pricking (84·5 per cent.). After an interval of 3 minutes the fertilization percentage halved and no fertilization occurred at longer intervals.

In sections of eggs fixed 2 minutes after pricking it can be seen that the wave of granule discharge has not yet reached the equator (Text-fig. 1B); the granules in the region of the animal pole are still unchanged (Plate 2, fig. B). After 3 minutes this wave has already spread over the animal region (Text-fig. 1C), in some of the eggs reaching its top, while at the opening of the terminal canal of the micropyles the contact between cytoplasm and membrane is still preserved (Plate 2, fig. C); in other eggs the cortical reaction is finished and the membrane separated (Plate 2, fig. D). It seems that in the first case the spermatozoon is still able to penetrate the egg, while in the second it is already impossible. Four minutes later the separation of membranes is completed in all cases (Text-fig. 1D; Plate 2, fig. E).

TABLE 4

Experiments on pricking the vegetative region of sturgeon eggs at various temperatures

+ eggs fertilizable. — fertilization impossible

Time from pricking to insemination	*Temperature (°C.)*		
	13·8	14·5	22·0
(minutes)			
1	+	+	+
2	+	+	—
3	+	—	—
4	—	—	—
5	—	—	—

The remaining experiments gave similar results. On pricking the vegetative region at intervals of 5, 10, 20, and 60 seconds, 100 per cent. of eggs were fertilized in some aliquots. The loss of fertilizability in different experiments took place at a different time after pricking: the higher the temperature the earlier the loss (Table 4). We can see convincingly in sections that the moment of the loss of fertilizability coincides in all cases with the secretion of cortical granules by the cytoplasm adjacent to the micropyles.

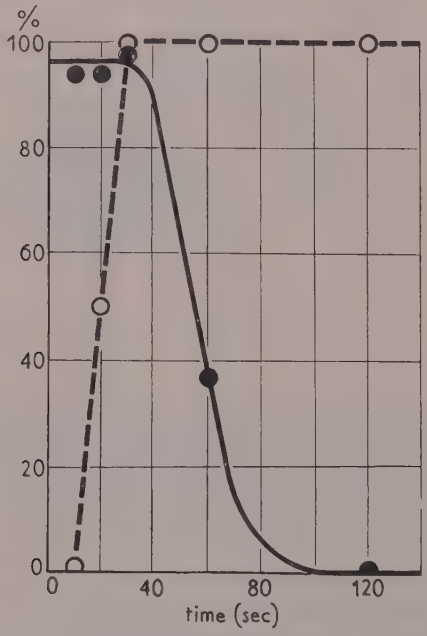
Thus in all experiments no decrease of fertilizability was observed for a long time after pricking in the region of the vegetative pole (up to 1–2 minutes); on the other hand, the loss of this ability coincided precisely with the completion of the cortical reaction and the separation of membranes in the micropylar region.

(*b*) *Activation of trout eggs by water treatment*

In the experiments with trout eggs water was used as an activating agent instead of pricking. Trout eggs were inseminated at different times after putting

them into water; simultaneously, other egg aliquots were transferred from water into Ringer and 0·1 M versene solutions to reveal the onset of early activation changes. It is known that salmonid eggs are not activated in Ringer solution (or in 0·1 N sodium chloride) but that activation once started continues to develop (Kusa, 1950; Devillers, Thomopoulos, Colas, 1954; Zotin, 1954). Activation also fails in 0·1 M versene solution (Dettlaff, 1959).

The experiment was carried out twice. It turned out (Text-fig. 2) that 10 seconds after putting into water the eggs were not yet activated, 20 seconds later half of them were activated, while all were activated after 30 seconds: if these eggs are put into Ringer and versene solution, a perivitelline space of normal size, the zone of fat droplets, and the blastodisc are formed. At the same time on insemination 30 seconds after putting the eggs into water all of them can still be fertilized. Fertilizability rapidly decreases after 60 seconds and is completely lost after 120 seconds.



TEXT-FIG. 2. The loss of fertilizability in trout egg with time after activation by immersion into water (sperm density on insemination $1{\cdot}63 \times 10^7$/m). Continuous line, percentage of fertilized eggs; dotted line, percentage of eggs which proved to be activated when put into Ringer solution.

A study of sections of 53 eggs fixed at different time intervals after they were put into water (temperature 4·0° C.) showed that the cortical egg layer preserved for 40 seconds the same appearance that it had prior to immersion (Plate 3, fig. 1A). After 60 seconds the layer of cortical alveoli remains still unchanged in

some of the eggs (Plate 3, fig. 1B), while in others the breakdown of alveoli has begun. After 80 seconds an intense secretion process was observed in the micropylar region in all cases: the alveolar wall adjacent to the surface burst and the colloidal content was released under the egg membrane, where it could be readily distinguished on staining with Heidenhain's azan method in the form of light blue clots usually adhering to the inner surface of the membrane (Plate 3, fig. 1C). 120 seconds after the immersion of eggs in water the process of breakdown of cortical alveoli had spread further, while a smoothing of the cytoplasm surface and a solution of the released content of the alveoli had started in the micropylar region (Plate 3, fig. 1D).

The data obtained are in good agreement with the observation made by Kusa (1950) that eggs of the dog salmon (*Oncorhynchus keta*) when put into water (temperature 10° C.) lost their fertilizability in 120 seconds; but these data are at variance with those of K. Yamamoto (1951), also obtained on the dog salmon, according to whom the eggs in water ceased to undergo fertilization only after 30 minutes (temperature 5–7° C.).

Thus no decrease of fertilizability is observed in trout eggs during a considerable time after the application of the activating agent. Thirty seconds after immersion in water some initial changes (that seem to correspond to the fertilization impulse) have already occurred in eggs, since they behave in Ringer and versene solutions like activated ones. However, the alveoli are unchanged at this time and the spermatozoon enters the egg despite the initiated change. The fertilizability is lost only at the moment of breakdown of cortical alveoli in the micropylar region

3. *Blocking action of perivitelline fluid*

(*a*) *Insemination of activated trout eggs after the removal of perivitelline fluid*

After the completion of the cortical reaction the content of the alveoli passes into the perivitelline fluid. If it is just this secretion that creates a barrier preventing the entering of supernumerary spermatozoa, then polyspermic fertilization should occur in the absence of perivitelline fluid. In order to examine this suggestion, trout eggs were activated by immersion in water, an aperture (diameter *c.* 2 mm.) was then cut in the vitelline membrane (chorion) through which the end of a pipette was introduced into the space between the egg surface and the membrane, and perivitelline fluid was washed out with a stream of water. The sperm then was introduced under the chorion by means of a pipette. Insemination was carried out from 25 to 120 minutes after immersion of the eggs into water. Untreated eggs that were simultaneously inseminated served as control. The results are presented in Table 5 (experiment 1). It can be seen that fertilization of activated eggs is possible even 120 minutes after the application of the activating agent.

The cleavage of those eggs that became fertilized shows features characteristic of polyspermy. In some cases on the periphery of the blastodisc some small

blastomeres separate, while the bulk of the cytoplasm remains undivided. In other cases the whole blastodisc divides into many blastomeres (Plate 3, fig. 2B), while control eggs are at the same time at the stage of the second division (Plate 3, fig. 2A). The cleavage pattern of such eggs is very similar to that observed after experimental polyspermy in frogs (Brachet, 1910) and sturgeon (Ginsburg, 1953; Dettlaff & Ginsburg, 1954). In sections of trout eggs fixed 6 hours after insemination (temperature 8·3–8·7° C.) up to 9 pronuclei were found.

TABLE 5

Fertilization of activated trout eggs (with aperture in the membrane)

No. of experiment	*Mode of insemination*	*Interval between egg immersion into water and insemination*	*Total number of eggs*	*Fertilized eggs*	
		(minutes)			(%)
(1)	Washing out perivitelline fluid and introduction of sperm under the membrane	25–45	33	23	69·7
		60	59	32	54·2
		120	37	21	56·8
(2)	Introduction of sperm into the water	20–45	16	0	0
		60, 120	18	0	0

In the second experiment an orifice in the chorion was also cut but the perivitelline fluid was not washed out, while insemination was carried out by introduction of a large amount of sperm into the water. No fertilization took place (Table 5).

Thus the surface layer of the activated trout egg becomes accessible to spermatozoa after removal of the perivitelline fluid.

(*b*) *The action of perivitelline fluid on spermatozoa*

Fertilized or activated trout eggs were superficially dried on filter paper, their chorion was pricked and perivitelline fluid removed by means of a fine glass pipette. If a drop of this fluid was mixed with a drop of trout sperm, an energetic agglutination of spermatozoa was observed (Plate 3, fig. 3A).[1]

Perivitelline fluid caused agglutination from the earliest time it was procured, i.e. 20 minutes after egg activation. After 24 hours this effect had not decreased and had even increased (clearly marked agglutination was observed on 1 : 4 dilution of this perivitelline fluid in water, while no agglutination took place after such dilution of earlier fluid). It can be seen in the smears of agglutinated sperm that the sperm tail becomes sticky and forms characteristic loops (cf. Plate 3, figs. 3 B, C).

DISCUSSION

The penetration of the spermatozoon into activated sturgeon and trout eggs becomes impossible at the moment when the cortical granules (the contents of

[1] In autumn 1960 the sperm-agglutinating agent in the perivitelline fluid of trout was shown to be inactivated by trypsin (by 0·1 per cent. trypsin solution in 1 per cent. $NaHCO_3$ in 15–20 seconds and by 0·001 per cent. solution in 6 minutes at 4.0° C.) and by boiling (in 1 hour–1 hour 40 minutes). These data suggest that the agglutinating agent is protein in nature.

the alveoli) are secreted in the micropylar region. On the other hand, retardation of the discharge of cortical granules in sturgeon eggs pretreated with urethane, ethyl ether, or acetone is accompanied by an increased incidence of polyspermy. These facts indicate the existence of a causal connexion between the discharge of substances contained in the cortical structures and the block to polyspermy. The results obtained are in good agreement with several observations made on sea-urchin eggs; these latter show that when the discharge of cortical granules is inhibited, or even retarded, then fertilization is polyspermic (Moore, 1916; Okazaki, 1956; Hagström & Allen, 1956; Hagström, 1957; Perlmann & Hagström, 1957). The same was observed in experiments involving the inhibition of cortical alveolar breakdown in the dog salmon (Kanoh & Yamamoto, 1957).

The role in the block to polyspermy assigned to the substances contained in the cortical granules (alveoli) which, after the latter break down, pass into the perivitelline fluid, finds support also in the experiments where the removal of these substances renders an egg already fertilized or activated accessible to spermatozoa. This was shown in the experiments described above on the washing out of perivitelline fluid in activated trout eggs. It seems that the results obtained on the Pacific herring (*Clupea pallasii*) should be interpreted in the same sense. Yanagimachi (1957) removed the egg membrane in Pacific herring mechanically, partially cutting it with iridectomy scissors and forcing the egg out through the aperture formed. On inseminating such denuded eggs, both non-fertilized and previously fertilized or activated by pricking, the penetration of numerous spermatozoa was observed, accompanied by an atypical cleavage characteristic of polyspermy. T. S. Yamamoto (1958) dissolved the membrane of herring eggs by a double treatment with acidified Ringer's solution and 0·2 per cent. trypsin in Ringer's solution. On insemination of these naked eggs polyspermic fertilization was observed, probably because perivitelline fluid was not retained at the egg surface in the absence of the chorion.

Similar results were obtained on sea-urchin eggs. Nakano (1954) mechanically removed the membrane of *Hemicentrotus pulcherrimus* eggs activated with saponin solution; thereafter these eggs could undergo fertilization (which was, as a rule, polyspermic).

Tyler, Monroy, & Metz (1956) mechanically removed the membrane of fertilized eggs of *Lytechinus pictus* and *L. variegatus*; on re-insemination polyspermic fertilization was observed. However, in experiments on other sea-urchin species mechanical de-membranation was insufficient for re-fertilization. It was obtained only after the application of treatments that dissolved the hyaline layer: treatment with Ca- and Mg-free media or urea solution (Sugiyama, 1951; Hagström & Hagström, 1954; Nakano, 1956). After treatment of activated *M. pulcherrimus* eggs with urea solution their fertilizability appreciably increased (Nakano, 1954).

Since the hyaline layer dissolved in these experiments is an extracellular

structure (Harvey, 1934) and seems to be formed at the expense of substances previously contained in cortical granules and then passed to the perivitelline fluid (Parpart & Cagle, 1957; see also Allen, 1958), the results obtained in the experiments on fish and sea-urchin eggs appear to be in principle similar: in both cases the block to polyspermy is eliminated after the removal of polysaccharide-containing derivatives of cortical granules (alveoli).

The detection of the agglutinating action of perivitelline fluid on spermatozoa, which is in good agreement with the analogous data of Motomura for sea urchin (see Metz, 1957), points to the probable mechanism of the prevention of polyspermy. It should be borne in mind, however, that, along with the substances previously contained in cortical structures, other secretions of the egg can enter the perivitelline fluid (as shown, for example, for Acipenseridae by Dettlaff & Ginsburg, 1954; Dettlaff, 1957). Therefore the effect obtained cannot be unreservedly ascribed to the action of the content of the alveoli, though the role played by the discharge of these substances in the block to polyspermy, which was found in other experiments, makes this interpretation seem the most likely.

During the period from the moment of the application of the activating agent until the completion of the cortical reaction in the micropylar region, in both sturgeon and trout, no decrease of the ability of the egg cytoplasm to accept the spermatozoon was observed. This tells against the existence in fish of any rapidly propagated block to polyspermy connected with invisible changes (fertilization impulse). Here the block to polyspermy seems to be realized only by means of the discharge of cortical granules (content of alveoli).

The conception of the diphasic block to polyspermy in sea urchins remains so far a hypothesis. The experiments with 'partially fertilized' sea-urchin eggs (Allen & Hagström, 1955 *a*, *b*; Hagström & Runnström, 1959) tell against the existence of any stable block preceding the cortical reaction. In these experiments the cortical reaction was interrupted several seconds after the fast block should have been established, but on re-insemination spermatozoa easily penetrated such eggs through that part of the surface which preserved cortical granules.

If the hypothesis of a diphasic block is nevertheless proved by further thorough study of the early developmental stages of sea-urchin eggs, then the difference in the polyspermy-preventing mechanism in echinoderms and fish should be interpreted in relation to the different modes of sperm–egg collision in these animals. In fish spermatozoa penetrate into the egg only in strictly localized sites, through micropyles. Consequently the blocking action of the discharged content of cortical structures is sufficient to prevent polyspermy. Salmonid eggs possess but one micropyle. The width of its terminal canal is such that the fertilizing spermatozoon fills the whole aperture and, until it is drawn into the cytoplasm, the penetration of other spermatozoa is physically impossible; but by then the breakdown of cortical alveoli is already accomplished.

Sturgeon eggs have several micropyles and hence the possibility of the penetra-

tion of several spermatozoa exists. Since micropyles are located in a group on a small area of the egg surface, and as the cortical reaction extends over this area in a very short time, a considerable incidence of polyspermy can occur only on insemination with very dense sperm suspensions, as has been shown experimentally. In nature such density can occur only as a rare event, since sturgeon spawn in rapid current where spermatozoa are scattered by the water stream.

Unlike fish, the whole egg surface in Echinoderms is accessible to spermatozoa, which may therefore require a more complicated defence mechanism against polyspermy.

SUMMARY

1. Treatment of sturgeon eggs with the polyspermy-inducers urethane, ethyl ether, and acetone prolongs the latent period of the cortical reaction and apparently retards the actual secretion of the cortical granules.

2. On activation of sturgeon eggs by pricking and of trout eggs by immersion in water, they lose their fertilizability at the moment of the discharge of cortical granules (the content of the alveoli) in the micropylar region. Before this no decrease in ability of the egg cytoplasm to accept a spermatozoon is observed.

3. After removal of the perivitelline fluid (containing substances discharged from the cortical alveoli) penetration of numerous spermatozoa into activated trout eggs becomes possible.

4. Perivitelline fluid of trout causes an agglutination of spermatozoa.

5. The data obtained provide evidence that the block to polyspermy in fishes is a one-step process realized by means of the discharge of cortical granules (the content of the alveoli).

Блокирование полиспермии у осетра и форели и роль кортикальных гранул (альвеол) в этом процессе.

А. С. Гинзбург

Выводы

1. При воздействии на яйца осетра раствором уретана, парами этилового эфира и ацетона (агентами, стимулирующими полиспермию) наблюдается удлинение латентного периода кортикальной реакции и, повидимому, некоторое замедление самого процесса выделения кортикальных гранул.

2. При активации яиц осетра уколом и яиц форели водой они утрачивают способность к оплодотворению в момент выделения кортикальных гранул (содержимого альвеол) в области микропиле. До этого не наблюдается какого-либо снижения способности цитоплазмы яйца к восприятию спермия.

3. После удаления перивителлиновой жидкости (содержащей вещества, выделенные из кортикальных альвеол) возможно множественное проникновение спермиев в активированные яйца форели.

4. Перивителлиновая жидкость форели оказывает на спермии агглютинирующее действие.

5. Полученные данные свидетельствуют о том, что блокирование полиспермии у рыб осуществляется одноактно, посредством выделения кортикальных гранул (содержимого альвеол).

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EXPLANATION OF PLATES

Key to abbreviations

CA, cortical alveoli; *CCA*, colloidal content of cortical alveoli discharged from the egg; *CG*, cortical granules; *FA*, funnel-shaped aperture of micropyle; *J*, jelly coat; *M*, metaphase of the second maturation division; *PG*, pigment granules; *SV*, large-sized single vacuoles; *TC*, terminal canal of micropyle; *V*, vacuoles in the cortical layer; *VM*, vitelline membrane (in sturgeon external and internal vitelline membranes; the border between them is indistinguishable on photomicrographs; in trout the vitelline membrane is also called chorion). *YG*, yolk granules.

Plate 1

Changes in cortical reaction in sturgeon eggs pretreated with polyspermy inducers. Egg sections in micropylar region. Fixation with Sanfelice fluid, staining with Heidenhain's Azan.

Fig. A. Non-fertilized ripe egg.
Fig. B. Control (untreated egg), 10 seconds after insemination.
Fig. C. Urethane pretreatment, 10 seconds after insemination.
Fig. D. Pretreatment with acetone vapour, 10 seconds after insemination.
Fig. E. Control, 20 seconds after insemination.
Fig. F. Urethane pretreatment, 20 seconds after insemination.
Fig. G. Pretreatment with ethyl ether vapour, 20 seconds after insemination.
Fig. H. Pretreatment with acetone vapour, 20 seconds after insemination.

Plate 2

Cortical reaction in micropylar region of sturgeon egg following activation by pricking. Fixation with Sanfelice fluid, staining with Heidenhain's Azan.

Fig. A, 5 seconds after pricking into animal region; Figs. B, 2 minutes; C and D, 3 minutes; E, 4 minutes after pricking into vegetative region.

Plate 3

Fig. 1. Cortical reaction in micropylar region of the trout egg activated by contact with water. Fixation with Sanfelice fluid, staining with Azan. A, non-treated egg; B, 60 seconds, C, 80 seconds; D, 120 seconds after immersion into water.

Fig. 2. Blastodisc of the trout eggs. A, control egg at the stage of the second cleavage division; B, an egg inseminated through an aperture in its membrane 25 minutes after immersion in water.

Fig. 3. Trout spermatozoa. Fixation with the vapour of osmium tetroxide, staining with Heidenhain's iron haematoxylin. A, a drop of sperm after the addition of perivitelline fluid taken 40 minutes after egg activation; B, a smear of agglutinated spermatozoa; C, control, a smear of sperm diluted with Ringer solution.

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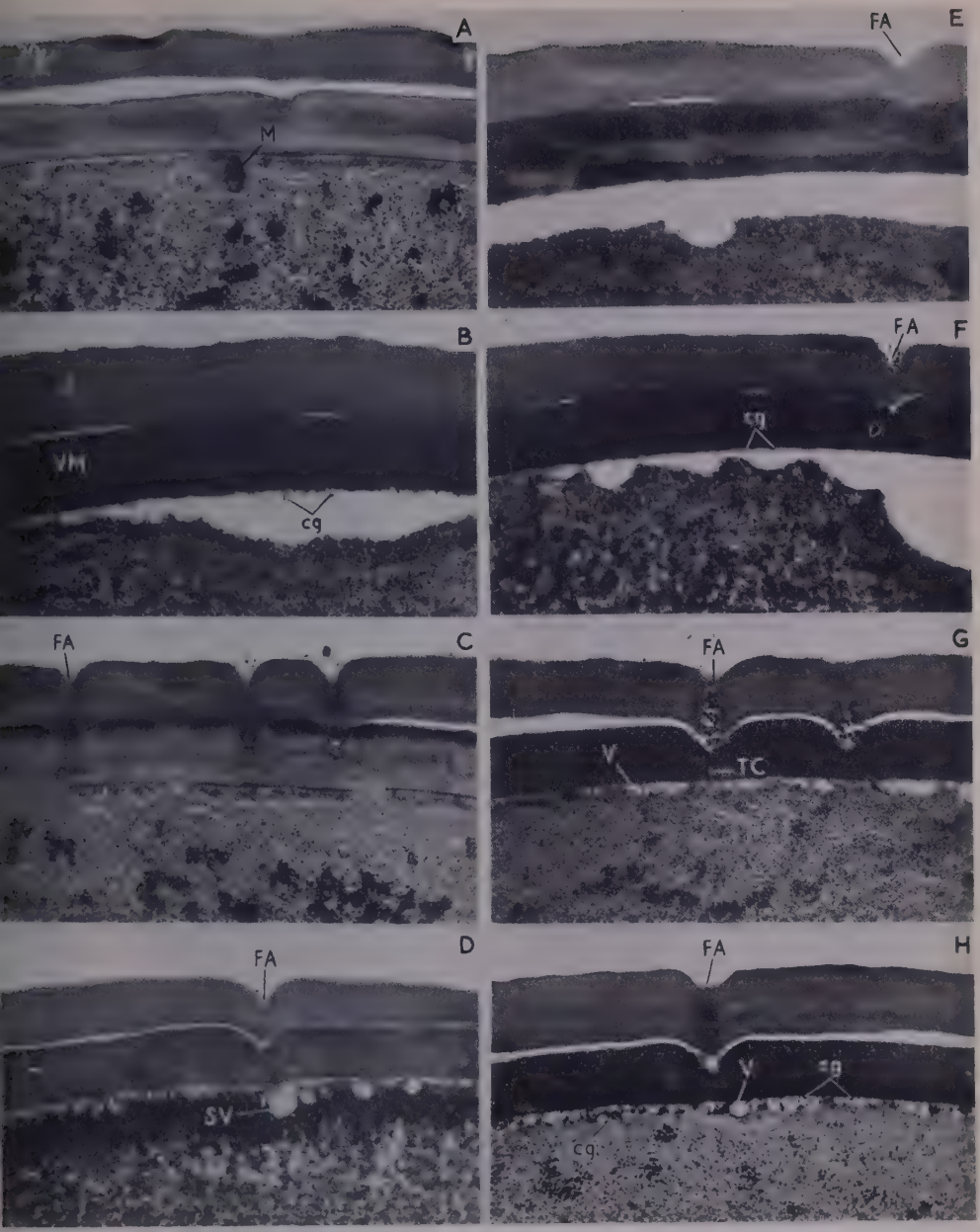

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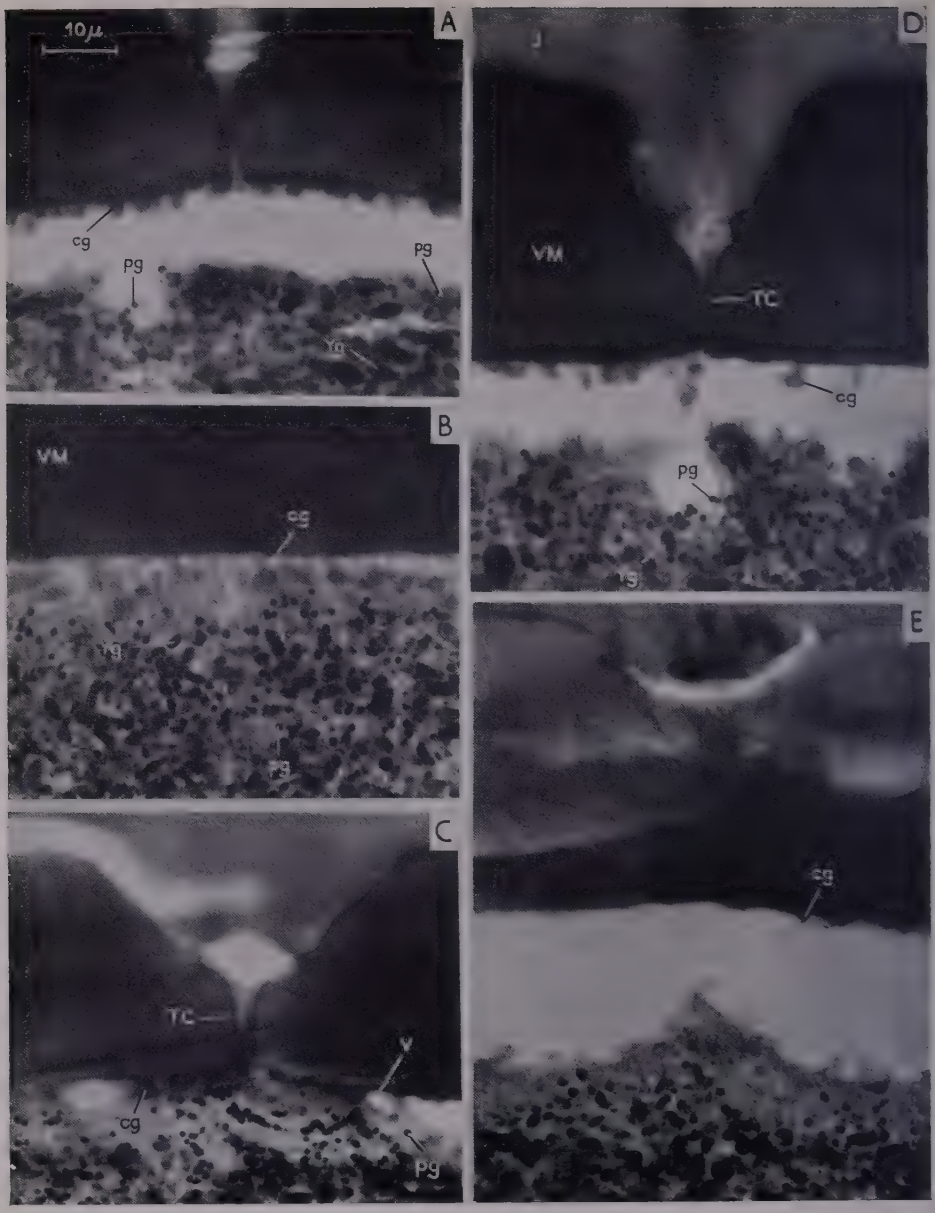
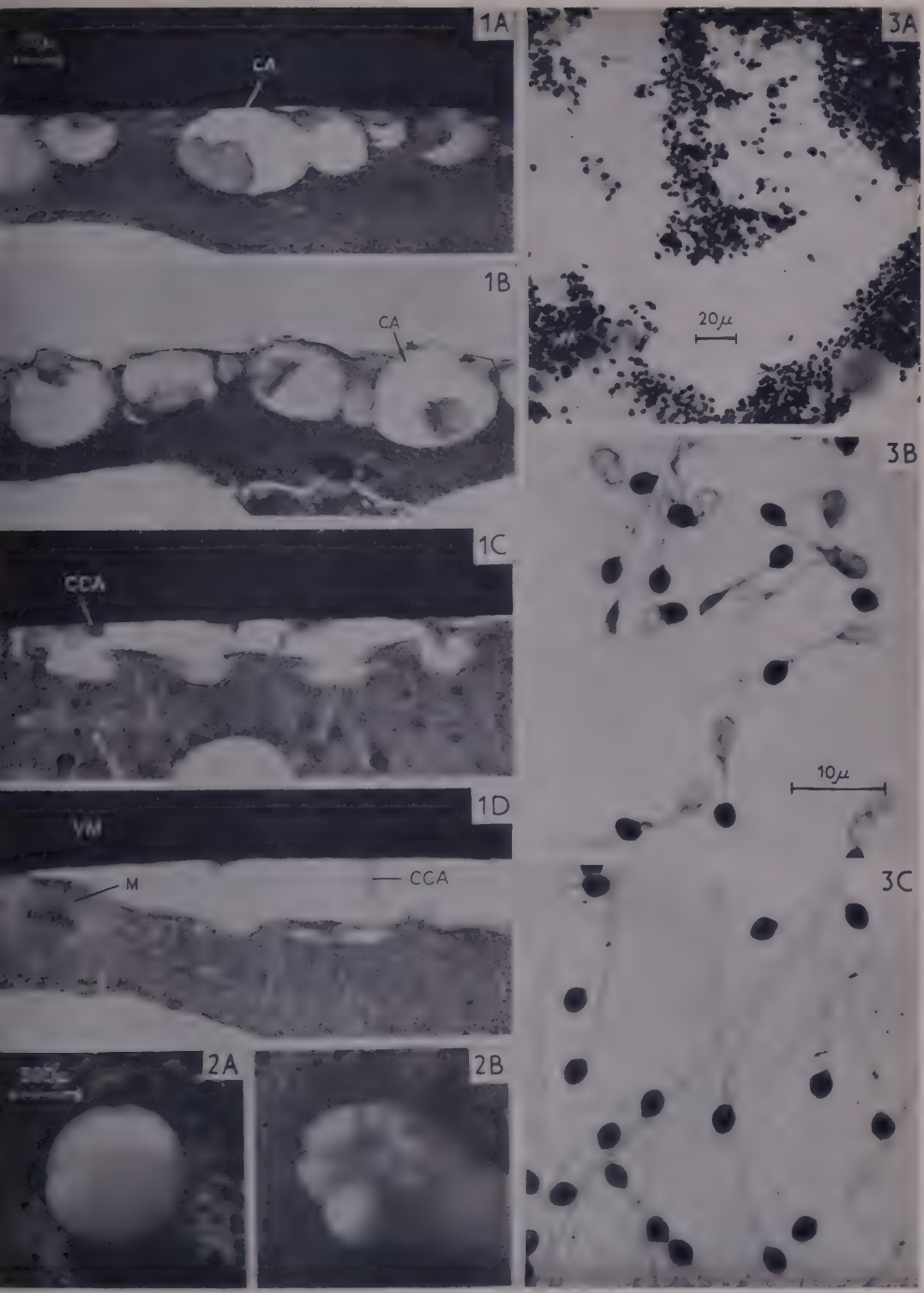
Plate 1



A. S. GINSBURG

Plate 2



A. S. GINSBURG

Plate 3

An Immuno-embryological Study on the Chick Lens

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WITH FIVE PLATES

INTRODUCTION

It was shown in previous experiments that the lens of the adult chick contains at least seven substances capable of acting as antigens (Langman, 1959). When lens extracts of chick embryos of various ages were analysed with the same technique, it was found that the antigens present in the adult lens arise gradually in the course of development, preceding or coinciding with the appearance of new morphological structures. Similar results obtained by application of Oudin's technique (1948) have recently been reported by Konyukhov & Lishtvan (1959 *a*, *b*).

Though in our experiments and those of other workers (Rao, Kulkarni, Cooper, & Radhakrishnan, 1955; François, Wieme, Rabaey, & Neetens, 1955; Halbert, Locatcher-Khorazo, Swick, Witmer, Seegal, & Fitzgerald, 1957; Koniukhov & Lishtvan, 1959 *a*, *b*) the presence of 5–10 lens antigens has been demonstrated by means of immunological techniques, it has been difficult to correlate these findings with data previously obtained by chemical and electrophoretic methods. When Woods & Burky (1927) and Krause (1932, 1933) analysed the soluble lens proteins by means of iso-electric precipitation, three fractions were found, referred to as alpha crystallin (precipitated at pH = 5·2), beta crystallin (precipitated at pH = 7·2), and gamma crystallin (precipitated with ammonium sulphate). Smelser & von Sallmann (1949) and François *et al.* (1953, 1954), using paper-strip electrophoresis, similarly found three protein fractions. The fastest-moving component in the electrical field was shown to be identical to chemically prepared alpha crystallin.

In an attempt to correlate precipitin bands found by immunological methods with those observed by paper-strip electrophoresis, François *et al.* (1956) reported that alpha crystallin was represented in the agar plate by one precipitin line, while the other fractions had two corresponding precipitin bands each. Three additional precipitin bands found in the agar plate, however, could not be related to any electrophoretic fraction.

The present study was undertaken to correlate precipitin bands, found by

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testing lens extracts with lens antiserum in the agar plate (Ouchterlony method), with lens fractions isolated by means of continuous-flow electrophoresis and to examine the order in which these fractions arise during organogenesis. Finally, the molecular weight of the isolated lens fractions was estimated and correlated with their appearance during embryonic development.

MATERIALS AND METHODS

Antigens

Lenses from adult chick and embryos of 60, 72, and 96 hours, 6, 8, and 10 days were carefully dissected free from capsule and surrounding tissues and homogenized to a concentration of 100–250 mg. wet weight per ml. saline. After centrifugation for 10 minutes at 3,000 rev./min. the supernatant containing 1·5 per cent. protein was used for the tests.

Antibodies

A 25 per cent. adult lens extract was suspended in Freund's adjuvant (Difco Bacto-adjuvant, complete) in a ratio of 3:2 and injected subcutaneously at five widely separate sites into a number of rabbits. The amount injected at each site was 1 ml. This procedure was repeated 4–6 times at weekly intervals. The serum of the rabbits was obtained 10 days after the last injection and will be referred to as lens antiserum.

Agar-diffusion technique

Agar plates were prepared as previously described (Langman, 1959) with 2 per cent. dialysed filtered agar (Difco; B 140; pH 7·2) to which 0·01 per cent. merthiolate had been added. Peripheral wells were made at a distance of 6–20 mm. from the central well and the tests were carried out at 4° C.

Paper-strip electrophoresis

Solutions of 10–25 per cent. lens extracts in saline were analysed in the Spinco Model R paper electrophoresis cell using Veronal buffer at pH 8·6 and ionic strength 0·02. After staining with 1 per cent. Bromphenol blue, the paper strips were analysed with the Spinco Analytrol Model R.B.

Continuous-flow electrophoresis

Adult lens extract containing 15 mg. protein per ml. was run in the Spinco Model C.P. continuous-flow electrophoresis apparatus for 36 hours. The analysis was made at 4° C. in Veronal buffer at pH 8·6 and ionic strength 0·02. The current used was 60 mA. and sample flow rate adjusted to deliver 3·2 ml. per hour. The lower curtain was equilibrated for 3 hours before the sample was applied. Fractions were collected, dialysed, and concentrated to a protein value of 35 ml./100 ml. and tested for homogeneity by means of paper electrophoresis and ultracentrifuge. At the completion of the separation, the curtain was dried at

120° C. and washed and stained with 1 per cent. Bromphenol blue for 30 minutes (Spinco No. 300–897, one gram Dye B-4 per litre purified Methanol).

Ultracentrifuge analysis

Total lens extract and isolated fractions obtained by means of continuous-flow electrophoresis (Protein concentration 0·38–0·82 mg. per cent.) were analysed at pH 8·6 in a Spinco Model E ultracentrifuge at a speed of 60,000 rev./min. at 20° C.

RESULTS

Analysis of adult lens extract

When a 25 per cent. lens extract was tested with lens antiserum during a 14-day period, the first precipitin band appearing between the antigen well (top) and antibody well (bottom) became visible at the 3rd day of the diffusion test (Plate 1, fig. 1). The antigen causing this band will be referred to as Fraction II and its precipitin line as 'band Fraction II'. An additional rather broad and vague band was apparent on the 4th day (referred to as 'band Fraction III'), while on day 5 of the test a third precipitin line became visible ('band Fraction I') (Plate 1, figs. 2, 3). During the succeeding days of the test, 'band Fraction II' split up into a number of well-defined lines, suggesting an equal number of closely related antigenic subfractions. In addition, it was observed that the bands of Fraction II moved through the agar in the direction of the antibody well and showed a curvature convex to the antigen well (Plate 1, figs. 4, 5, 6). Similarly, 'band Fraction III' was seen to consist of two broad and poorly defined bands, curving around the antibody well. On the contrary, the precipitin line referred to as 'band Fraction I' was straight or slightly curved in the opposite direction, suggesting that the protein making up Fraction I had a molecular weight higher than that of Fractions II and III. It was concluded that the adult chick lens contains substantial amounts of at least 7 to 10 water-soluble antigenic components, grouped into three main fractions. While Fraction I contains only one antigen, Fraction II consists of at least four closely related subfractions and Fraction III of two subfractions.

When a 10 per cent. lens extract was analysed by means of paper-strip electrophoresis, three bands were formed, referred to as Fraction A, Fraction B, and Fraction C (Plate 2, figs. 7*a*, 8). The fastest-moving component in the electrical field (Fraction A) was found to have the same mobility as material precipitated from total lens extract at pH 5·2 and is therefore identical to alpha crystallin. Fraction B showed the same mobility as material precipitated from total lens extract at pH 7·2 and is therefore considered to be identical to beta crystallin. Fraction C, having the same mobility as material obtained from lens extract by saturation with ammonium sulphate (after removal of the precipitates formed at pH 5·2 and pH 7·2), is considered to be identical to gamma crystallin.

When subsequently adult lens extract was run in the continuous-flow

electrophoresis and the fastest-moving fraction was collected, concentrated, and tested on the paper strip, it showed a mobility identical to that of alpha crystallin (Plate 2, fig. 7*b*). Similarly, when the two other fractions were isolated from the curtain after repeated runs and tested on the paper strip, they showed a mobility identical to that of beta and gamma crystallin respectively (Plate 2, figs. 7 *c*, *d*). Therefore, the various fractions isolated from the electrophoretic curtain are considered to be identical to alpha, beta, and gamma crystallin.

TABLE 1

Sedimentation constant of lens proteins

	Protein mg./100 ml.	pH	S_{20}
Total chick lens: 3 peaks Fraction A	0·3	8·6	17·75
Fraction B			9·506
Fraction C			4·244
Total chick lens: 3 peaks Fraction A	0·6	8·6	17·26
Fraction B			8·989
Fraction C			4·386
Isolated fractions:			
α-crystallin	0·51	8·6	17·74
β-crystallin	0·38	8·6	9·402
γ-crystallin	0·82	8·6	4·29

Lens extract analysed in the ultracentrifuge likewise showed the presence of three peaks, referred to as Fractions A, B, and C (Plate 3, fig. 9*a*). The sedimentation coefficient of Fraction A was found to be $(S_{20}) = 17{\cdot}2$–$17{\cdot}7$; of Fraction B $(S_{20}) = 8{\cdot}9$–$9{\cdot}5$; of Fraction C $(S_{20}) = 4{\cdot}2$–$4{\cdot}3$ (see Table 1). When the fastest-moving fraction collected from the curtain (alpha crystallin) was analysed in the ultracentrifuge, it formed one distinct single peak, indicating homogeneity of the material, and showed a sedimentation coefficient of $(S_{20}) = 17{\cdot}74$ (Plate 3, fig. 9*b*). This indicates that alpha crystallin is identical to Fraction A of the total lens extract. When similarly the two other fractions, beta and gamma crystallin, were isolated and tested in the ultracentrifuge, single peaks with sedimentation coefficients of $(S_{20}) = 9{\cdot}4$ and $(S_{20}) = 4{\cdot}2$ were found (Plate 3, figs. 9 *c*, *d*), indicating that Fractions B and C are identical to beta and gamma crystallin.

When alpha crystallin collected from the electrophoretic curtain was tested with lens antiserum, it formed one distinct precipitin line. Comparing this 'alpha crystallin band' with those formed by total lens extract (Plate 4, fig. 10), it was found to fuse with 'band Fraction I' and to cross with 'band Fraction II' (Plate 4, fig. 11)—a fact indicating that alpha crystallin is identical with Fraction I and non-identical with Fraction II. When beta crystallin was isolated from the

curtain and tested with lens antiserum, it formed a dense precipitin band, which fused with 'band Fraction II' of the total lens extract, indicating identity of beta crystallin and Fraction II (Plate 4, figs. 12, 13). Though during continuation of the test 'band Fraction II' split up in a number of precipitin lines, all these lines fused with the band formed by the isolated beta crystallin. Fig. 13 of Plate 4 shows that the precipitin band caused by isolated gamma crystallin fuses with 'band Fraction III', thus indicating identity. It was thus concluded that Fractions I, II, and III found by testing adult lens extract with lens antiserum are identical to alpha, beta, and gamma crystallin respectively.

Formation of alpha, beta, and gamma crystallin in the course of development

60-hour embryos (26–28 somites)

An extract prepared from one hundred 60-hour embryos and tested with lens antiserum in the agar plate formed one vague precipitin line. This '60-hour band' fused with the 'alpha crystallin band', indicating identity of the antigens (Plate 5, fig. 14). No additional bands were formed by the 60-hour extract although the concentration of the extract was varied and the test period prolonged. At this stage of lens development basophilic granules appear at the base of the placode cells (Plate 5, fig. 15), while acidophilic fibres are visible in the cytoplasm.

72-hour embryos (35–37 somites)

At this stage of development the placode has invaginated and formed a lens vesicle (Plate 5, fig. 17). The retina-facing cells of this vesicle show fibre formation. A lens extract prepared from 60 embryos of this age and tested with lens antiserum showed one vague and one dense band which in the periphery was split up in two bands (Plate 5, fig. 16). When comparing these bands with those of the adult lens, they were found to be identical to alpha and beta crystallin.

96-hour embryos (42–45 somites)

Lens extract prepared from 50 embryos and tested with lens antiserum showed the presence of two distinct bands, fusing with the alpha and beta crystallin bands (Plate 5, fig. 18). At this stage of development marginal fibre formation at the equator of the lens is apparent (Plate 5, fig. 19).

6-day embryos

When various concentrations of 6-day lens extract were tested with lens antiserum, the presence of two bands fusing with the alpha and beta crystallin bands respectively was recorded (Plate 5, fig. 20).

10-day embryos

Fig. 22 of Plate 5 shows the precipitin lines formed by a 10-day lens extract. It is evident that at this stage of development alpha, beta, and gamma crystallins are present. Fig. 23 shows the presence of nuclear and marginal lens fibres.

It was thus concluded that the various crystallins of the lens arise in consecutive order—that is, first alpha crystallin, secondly beta crystallin, and finally gamma crystallin.

Comparing the sedimentation coefficients of the crystallin fractions (see Table 1) with those of well-known proteins, it is assumed that the order of magnitude of the molecular weight of alpha crystallin is approximately 1,000,000; of beta crystallin approximately 200,000; and of gamma crystallin approximately 60,000. This seems to suggest that the first antigenic fraction which arises during development of the lens is of high molecular weight, while those detected at later stages of development are of lower molecular weight.

DISCUSSION

Coalescence of precipitin bands observed when two antigenic solutions are tested with the same antiserum indicates identity of antigens (Ouchterlony, 1953; Wilson & Pringle, 1955; Korngold, 1956). Thus, fusion of 'band Fraction I' with the band produced by electrophoretically isolated alpha crystallin indicates identity of the two substances (Plate 4, fig. 10).

To obtain additional evidence, alpha crystallin was prepared by iso-electric precipitation (Krause, 1933) and tested with lens antiserum. Fusion of the 'alpha crystallin band' and 'band Fraction I' was observed. Finally, alpha crystallin of the rabbit lens, made available to us by Dr. D. C. Wood (see Wood, Massi, & Solomon, 1959), was tested with lens antiserum. This material likewise produced a precipitin band, which showed coalescence with 'band Fraction I'. It has been concluded, therefore, that Fraction I is identical to alpha crystallin.

Examining the self-differentiation capacity of the chick lens McKeehan (1953, 1954) found that a lens placode of a 21-somite embryo is capable of independent lens formation when transplanted into the coelomic cavity of another embryo. At this stage of development the lens placode cells are believed to possess the basic chemical inventory required for lens differentiation. From our experiments it appears that alpha crystallin is the first lens antigen detectable during organogenesis—that is, at the lens placode stage. It seems therefore that alpha crystallin is a protein 'essential' for lens formation. Indeed, when lens extracts of representative species of mammals, birds, reptiles, amphibians, and fishes were tested with chick-lens antiserum, it was found that alpha crystallin is the only antigenic component to be present in the lens of the species examined. This indicates that alpha crystallin is distributed throughout the vertebrate series and does not possess strong species-specific properties. Beta and gamma crystallin were found to be present only in the more closely related classes, but not in man, mammals, and fish, indicating more specialized characteristics (Maisel & Langman, in press). When likewise various tissues of the chick were examined in the presence of lens antigens, it was found that alpha crystallin was one of the main antigenic components to be present in iris, retina, and cornea—that is, in those tissues which have the capacity to form a lens upon removal of the original lens (Lang-

man & Prescott, 1959; Van Deth, 1939). Thus, alpha crystallin is the first antigenic lens fraction detectable during lens development; it is found widespread throughout the vertebrate series and is present in those tissues which show lens regeneration capacity. It is therefore felt that the protein alpha crystallin plays an 'essential' role in lens formation.

When beta crystallin was collected from the electrophoretic curtain and analysed by paper-strip electrophoresis, it was found to be contaminated with a small amount of gamma crystallin. Only repeated re-running of the material in the continuous-flow electrophoresis apparatus made it possible to obtain a sample of beta crystallin showing a single peak in the ultracentrifuge. When this material was tested with lens antiserum in the agar-diffusion technique it showed the formation of one dense precipitin band. Only occasionally was the formation of two bands observed. Though the 4–7 precipitin lines formed by Fraction II of total lens extract fused with the 'beta crystallin line', thus indicating antigenic identity, it is surprising that electrophoretically isolated beta crystallin never formed more than one or two precipitin bands. This raises the question whether the multiplicity of precipitin lines formed by Fraction II are to be considered as artefacts, or whether the repeated treatment of the isolated beta crystallin has caused denaturation of some subfractions, thus leading to the formation of only one or two precipitin bands.

Thus far, it has generally been accepted that neither antigen nor antibody can diffuse beyond the precipitin zone, which acts as a barrier to this particular antigen-antibody system, while other antigens and antibodies go through (Ouchterlony, 1949, 1953; Wilson & Pringle, 1955; Korngold, 1956). In case of excess of an antigen the band moves by dissolution and reprecipitation in the direction of the antibody well. Recently, however, Kaminski (1954) and Korngold (1959) have suggested that excess of a single antigen may give rise to a second precipitin line. In such a case the originally formed band does not dissolve and move towards the antibody well, but a second band of the same antigen-antibody system is formed closely in front of the first one. This explanation does not seem to hold for our experiments since various observations indeed favour the existence of a number of closely related antigens in the beta fraction. When François *et al.* (1956) tested bovine lens extract by means of immunoelectrophoresis, two beta fractions were demonstrated. Firfarova (1956), using free electrophoresis, reported the presence of two to three beta fractions, while Resnik *et al.* (1959), analysing bovine lens extract by means of free boundary electrophoresis, suggested the presence of six closely related components in the beta fraction. An additional factor favouring the existence of a number of closely related antigenic substances in Fraction II is seen in the fact that the bands of Fraction II show a movement through the agar in the direction of the antiserum well in the course of the diffusion test. In cases of artefact the number of bands may increase (though never to the number observed for Fraction II), but movement of bands is difficult to explain. Based on these observations, it is

thought that beta crystallin consists of a number of closely related but distinct antigenic entities. Treatment with buffer and repeated running of beta crystallin in the continuous-flow electrophoresis apparatus is held responsible for the fact that isolated beta crystallin forms only one or two precipitin bands when tested with lens antisera.

When the species-specificity of Fraction II (beta crystallin) was tested, it was found that closely related birds, such as the turkey, have a similar number of subfractions to the chick. The duck, however, is slightly different from the chick in regard to the number of beta components, while the turtle has only one beta component in common with the chick. Mammals and fishes do not show the presence of Fraction II when tested with chick-lens antiserum. Through alpha crystallin was found throughout the vertebrate series, beta crystallin seems to be species-specific to a much greater extent. The great number of subfractions in Fraction II (beta crystallin) and the species-specific properties of this protein, seem to make it a complicated and highly specialized substance playing an essential role in lens-fibre formation. As such, beta crystallin might be the target for environmental factors, acting during the development of the lens. Indeed, virus particles as found in German measles seem to cause destruction of the nuclear lens fibres only if acting during formation of these fibres (Töndury, 1952)—that is, the time when synthesis and formation of the various beta subfractions occur.

SUMMARY

The purpose of this experiment was to analyse location and time of appearance of lens proteins during lens development.

1. Applying the agar-diffusion technique of Ouchterlony the adult lens was found to contain substantial amounts of at least seven soluble antigenic substances, grouped into three main fractions and referred to as Fractions I, II, and III. While Fraction I consists of one antigenic substance, Fraction II contains at least four components and Fraction III usually two components.

2. Fraction I appears to be identical to the protein alpha crystallin (m.w. approximately 1,000,000) as prepared by continuous-flow electrophoresis and tested on homogeneity and identity by ultracentrifugation and paper electrophoresis. It is detected in the epithelial cells of the lens placode before the appearance of any other lens antigens and is thus considered to be the first lens protein to arise during organogenesis. The importance of this protein for lens development is also shown by its presence in the lens of representative species of the vertebrate series and by its presence in iris, retina, and cornea—that is, those tissues which show the capacity to form a lens after removal of the original lens.

3. Fraction II appears to be identical to beta crystallin (m.w. approximately 200,000) and is characterized by at least four closely related antigenic subfractions, which appear at the onset of differentiation and growth of the nuclear lens fibres. As it is localized in the nucleus of the lens, the typical site of con-

genital cataracts, it is believed to be the main target for teratogenic factors such as German measles virus, which causes a cataract only if acting during differentiation and growth of the nuclear lens fibres.

4. Fraction III appears to be identical to gamma crystallin (m.w. approximately 60.000) and is the last lens antigen to arise during lens development. Its morphological position could not be determined.

RÉSUMÉ

Études immuno-embryologiques sur le cristallin du poulet

Le but de cette recherche expérimentale a été d'analyser la localisation et le moment d'apparition des protéines cristalliniennes pendant le développement du cristallin.

1. Par l'application de la technique de diffusion en agar d'Ouchterlony, il a été trouvé que le cristallin adulte contient des quantités appréciables d'au moins sept substances antigéniques solubles, groupées en trois fractions principales désignées I, II et III.

Tandis que la Fraction I ne comprend qu'une substance antigénique, la Fraction II contient au moins quatre constituants et la Fraction III en contient habituellement deux.

2. La Fraction I paraît être identique à la protéine cristallinienne alpha (poids moléculaire d'environ 1.000.000) telle qu'on la prépare par électrophorèse à flux continu en testant ensuite son homogénéité et son identité par ultracentrifugation et électrophorèse sur papier. Elle est décelable dans les cellules épithéliales de la placode cristallinienne avant l'apparition de tout autre antigène du cristallin, et peut donc être considérée comme la première protéine cristallinienne apparaissant pendant l'organogénèse. L'importance de cette protéine dans le développement du cristallin est aussi démontrée par sa présence dans des cristallins de divers Vertébrés représentatifs et par sa mise en évidence dans l'iris, la rétine et la cornée, c'est-à-dire dans ces tissus qui sont capables, après l'ablation du cristallin, de former ce même organe.

3. La Fraction II paraît être identique à la cristalline beta (p.m. environ 200.000) et est caractérisée par la présence d'au moins quatre sous-fractions antigéniques, lesquelles apparaissent au début de la différenciation et de la croissance des fibres cristalliniennes. Comme cette fraction est localisée dans le noyau du cristallin, siège typique de la cataracte congénitale, on la croit spécialement touchée par des facteurs tératogéniques, tels que le virus de la rubéole, lequel cause la cataracte seulement quand il agit pendant la différenciation et la croissance des fibres cristalliniennes.

4. La Fraction III paraît être identique à la cristalline gamma (p.m. environ 60.000) et est le dernier antigène cristallinien à apparaître pendant le développement du cristallin. Sa localisation morphologique n'a pas été déterminee.

ACKNOWLEDGEMENTS

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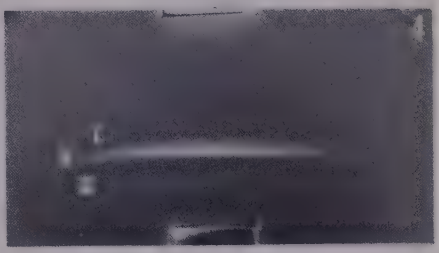

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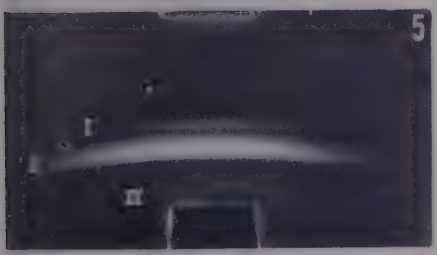

10 DAYS

14 DAYS

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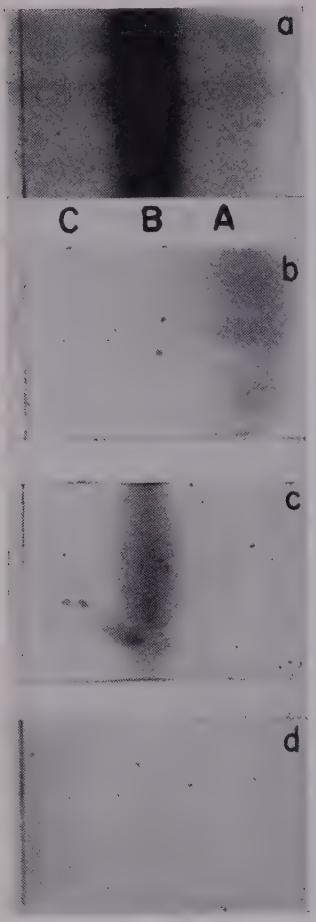
Plate 1



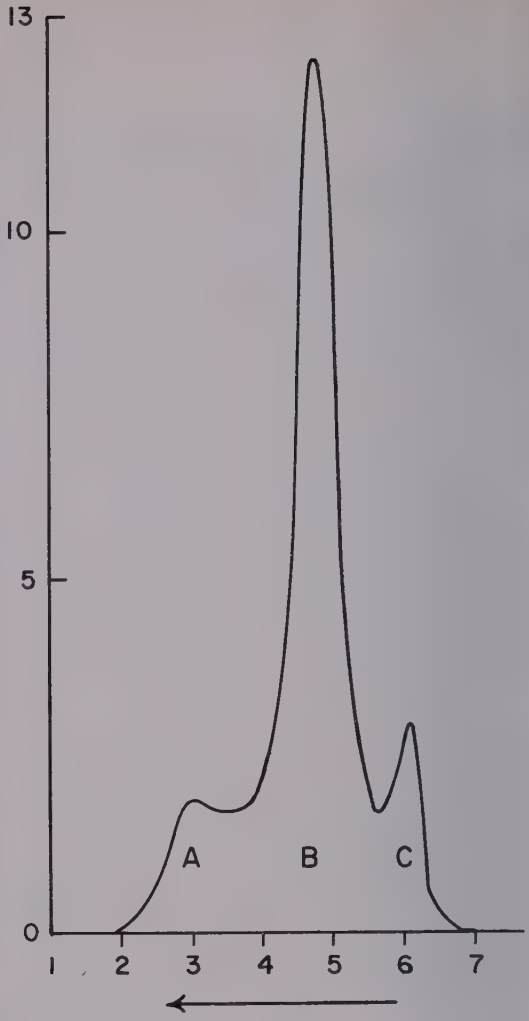


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Plate 2

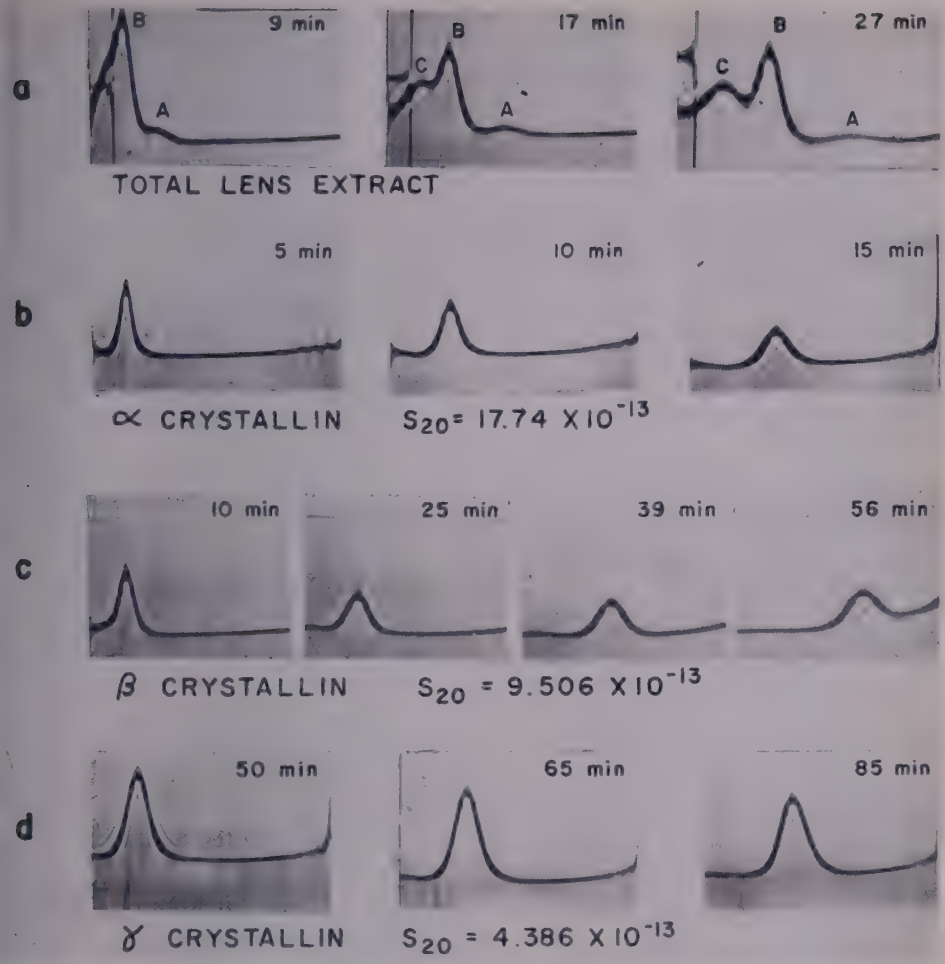

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Plate 3

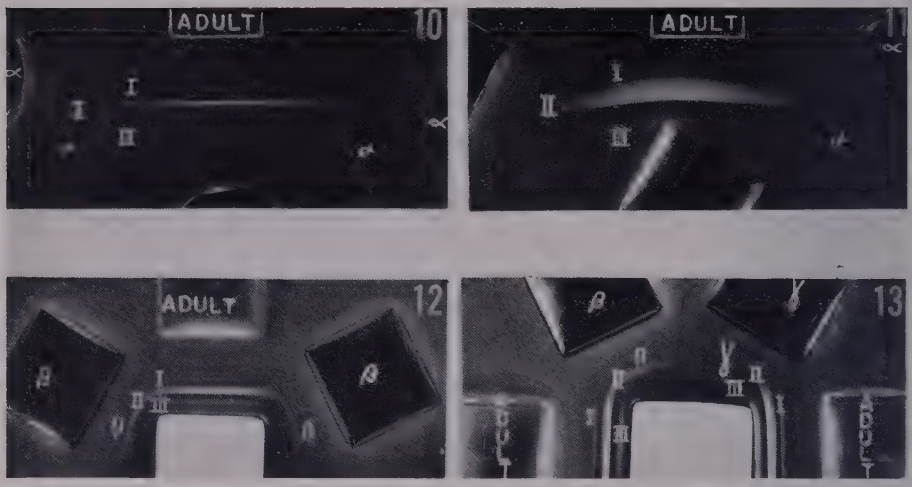

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Plate 4

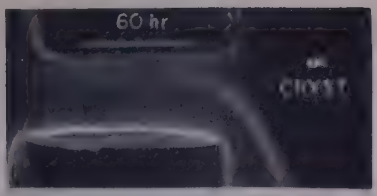

FIG 14

FIG 15 28 SOMITES

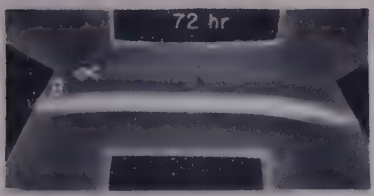

FIG 16

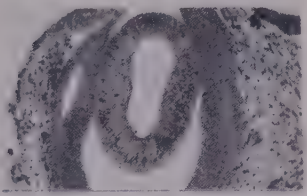

FIG 17 36 SOMITES

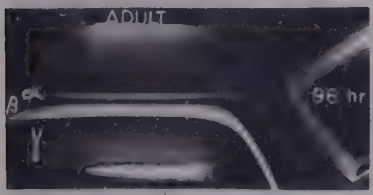

FIG 18

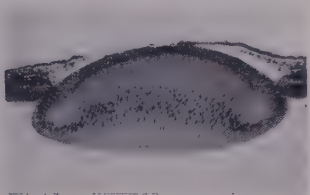

FIG 19 44 SOMITES

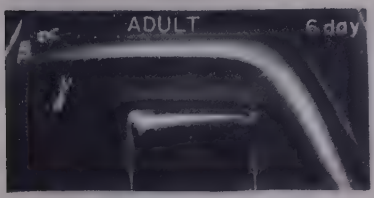

FIG 20

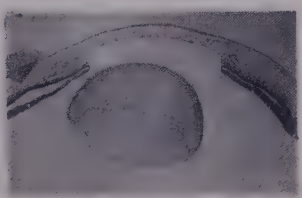

FIG 21 6 DAY

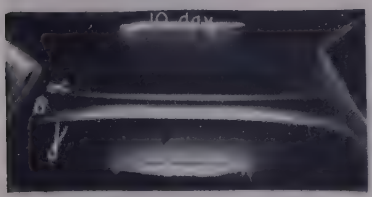

FIG 22

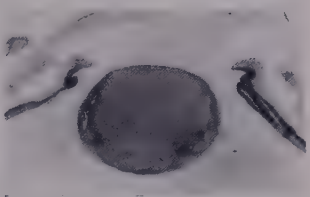

FIG 23 10 DAY

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Plate 5

Töndury, G. (1952). Zur Kenntnis der Embryopathia rubeolica, nebst Bemerkungen über die Wirkung anderer Viren auf den Keimling. *Geburtsh. u. Frauenheilk.* **12,** 865–88.

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EXPLANATION OF PLATES

Plate 1

Figs. 1–6. Appearance of precipitin lines found by testing 10 per cent. lens extract with lens antiserum by means of the agar-diffusion method. Top well contains antigens. Bottom well contains antiserum. Distance between wells 20 mm.; temperature 4° C. *Note*: (*a*) appearance of 'band Fraction II' at day 3, 'band Fraction III' at day 4, and 'band Fraction I' at day 5; (*b*) multiple bands of Fraction II at day 7 to 14; (*c*) curvature of 'band Fraction II' and 'band Fraction III' towards antibody well at day 7–14; (*d*) curvature of 'band Fraction I' towards antigen well at day 14; (*e*) movements of bands towards antibody well in the course of the test period.

Plate 2

Fig. 7. Pherogram showing: *a*, total lens extract; *b*, isolated alpha crystallin; *c*, isolated beta crystallin; *d*, isolated gamma crystallin.

Fig. 8. Electrophoretic pattern of total lens extract. Alpha crystallin is the fastest moving fraction.

Plate 3

Fig. 9. Ultracentrifugal analysis of lens extract and isolated lens fractions: *a*, total lens extract, note peaks *A*, *B*, and *C*; *b*, alpha crystallin; *c*, beta crystallin; *d*, gamma crystallin. Time indicated in minutes.

Plate 4

Fig. 10. Top well contains 10 per cent. adult lens extract; lateral wells contain alpha crystallin obtained from electrophoretic curtain; bottom well contains lens antiserum. Note coalescence of 'band Fraction I' and 'alpha crystallin band'.

Fig. 11. Note fusion of 'alpha crystallin band' with 'band Fraction I' and non-identity with 'band Fraction II'.

Fig. 12. Top well contains 10 per cent. adult lens extract; lateral wells contain beta crystallin. Note coalescence of 'beta crystallin band' with 'band Fraction II'.

Fig. 13. Lateral wells contain 10 per cent. adult lens extract. Left top well contains beta crystallin. Right top well contains gamma crystallin. Note coalescence of 'beta crystallin band' with 'band Fraction II' and coalescence of 'gamma crystallin band' with 'band Fraction III'.

Plate 5

Fig. 14. Top well contains 60-hour lens extract, lateral well contains alpha crystallin. Note coalescence of bands.

Fig. 15. Invaginating lens placode of 60-hour (28 somites) embryo.

Fig. 16. Precipitin bands observed by testing 72-hour lens extract with lens antiserum. Two dense precipitin lines can be seen in front of alpha crystallin band.

Fig. 17. Lens vesicle of 72-hour (36 somites) embryo.

Fig. 18. Top well contains 10 per cent. adult lens extract; lateral well contains 96-hour lens extract. Coalescence of '96-hour bands' with alpha and beta fractions can be noted.

Fig. 19. Lens of 96-hour embryo. Nuclear lens fibres have filled lumen of lens vesicle and cells in marginal zone at equator show beginning fibre formation.

Fig. 20. Note coalescence of precipitin bands formed by alpha and beta fractions with those formed by 6-day lens extract.

Fig. 21. Lens 6-day embryo showing marginal lens fibre formation.

Fig. 22. Precipitin lines formed by testing 10-day lens extract with lens antiserum. Note the presence of alpha, beta, and gamma precipitin bands.

Fig. 23. Lens 10-day embryo showing nuclear and marginal lens fibres.

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Development of Haemoglobin by De-embryonated Chick Blastoderms Cultured *in vitro* and the Effect of Abnormal RNA upon its Synthesis

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WITH TWO PLATES

INTRODUCTION

THE embryo provides a sequence of developmental stages in which proteins both structural and enzymatic appear or become detectable for the first time in a restricted group of dividing cells. The cells or tissues can be maintained *in vitro* for a period that may precede and include the synthesis of a specific 'cytoplasmic' protein. In this way systems of protein synthesis within the cells of higher organisms can be studied during those stages in which current hypotheses suggest that some structural code is passed on from the DNA of the nucleus to the cytoplasm where the synthesis of the protein becomes maximal.

Acellular preparations have contributed much to the elucidation of protein synthesis, but it is doubtful whether actual net synthesis has been obtained in systems less complex than the 'protoplast' developed by Spiegelman (1957). In order to study the synthesis of a specific protein it seems necessary at this stage to use whole cells. Micro-organisms for the most part have provided the cell system; however, Zamecnik *et al.* (1949), and Shimura *et al.* (1955) have studied the formation of silk protein in isolated silk gland. Hokin & Hokin (1956) and Daly, Allfrey, & Mirsky (1955) have used pancreatic tissue to synthesize specific enzyme proteins. Haemoglobin synthesis in reticulocytes by Boorsook *et al.* (1955) and Rabinovitz & Olson (1956) has provided another synthetic system.

The mechanism of protein synthesis is not clear and the part played by cytoplasmic RNA, nuclear RNA, and DNA is equally uncertain. There seems to be agreement that DNA-associated reactions initiate a synthetic pathway which leads by way of nuclear RNA to the ribonucleoprotein complexes in the cytoplasm which form a 'template' on which a specific protein is assembled (Gale & Folkes, 1955; Loftfield, 1957; Brachet, 1959; Marshak, 1959).

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This paper presents a study of certain aspects of haemoglobin production in the developing blood-islands of the early chick embryo differing in some respects from the tissue or cell systems mentioned above. The sequence of events, whatever they may be, leading to the appearance of haemoglobin in the cytoplasm of the embryonic erythroblasts may be conveniently separated in time by the culture of embryonic blood-islands at different developmental stages.

In vitro culture of blood-islands before, during, and after the appearance of haemoglobin allow the following assumptions to be made: (1) cells prior to the appearance of haemoglobin have not yet reached the stage of final cytoplasmic 'template' formation; (2) blood-islands in which haemoglobin is just detectable in scattered cells and small groups of cells have reached a stage when 'template' formation is probably just completed; (3) when haemoglobin is clearly present in the majority of cells the synthetic pathway has been established and is functioning efficiently.

With these assumptions in mind, the interference with RNA metabolism by metabolite analogues should give some information about the hypothetical 'template' RNA and its susceptibility to abnormal RNA. A comparable situation among micro-organisms is the relative inhibition of adaptive and constitutive enzymes by the RNA base analogue 8-azaguanine in *Staphylococcus aureus* (Creaser, 1956). In this case the formation of a new protein template was inhibited to a greater extent than that of an established template. In system (1), above, the haemoglobin represents a new protein and its template a new template, while in (3) above, the haemoglobin is a protein whose synthetic pathway has been established.

The RNA base analogue 8-azaguanine has been used in many ways, from a specific antimetabolite in bacterial metabolism to a general inhibitor or modifier of embryonic development. It is well established that 8-azaguanine readily penetrates metabolizing cells and in those cases investigated it has been incorporated into the RNA fraction to form an abnormal RNA. It is replaceable, in some systems, by the normal metabolite guanine with the subsequent formation of normal RNA. In the presence of both the normal base and its analogue the synthesis of normal RNA continues.

MATERIAL AND METHODS

The embryonic disk, after approximately 20 hours' incubation, was removed from the egg, washed free of yolk, and transferred to a watch-glass containing 'fluid' albumen according to the method described by New (1955). The saline in which the yolk was suspended for dissection and the blastoderm washed was unbuffered Panett–Compton saline with 1 per cent. glucose. The embryo was allowed to develop *in vitro* at 37·5° C. until it reached the stage of development required. The glass ring was then flooded with buffered Panett–Compton glucose saline and the blastoderm de-embryonated while still attached to the vitelline membrane. Glass needles and a small pipette were used for the

dissection. The de-embryonated blastoderm was separated from the vitelline membrane with a blunt or ball-tipped glass needle. The ventral surface of the blastoderm is uppermost and it is advantageous for observation and subsequent development if it is transferred to the agar medium in this position. The developing blood-islands tend to become obscured by the dorsal ectoderm so that direct observation is difficult from the dorsal aspect. Adherence to the agar surface was much firmer and the well-defined flattened form of the blastoderm more effectively maintained when the dorsal surface was in contact with the gel. The de-embryonated blastoderm was washed several times in buffered glucose saline of the same composition as that to which agar was added to make the final medium, a 1 per cent. agar gel. When inhibitors were included in the gel, the blastoderm was washed in a saline medium containing the same concentration of additive as the gel medium.

The developing system is made up of islands of haemopoietic mesoderm and coelomic mesoderm, a layer of dorsal ectoderm adherent to the agar, and an ill-defined layer of yolk-sac entoderm and yolk granules on the uppermost surface. The transfer of the blastoderm to an agar medium stops cell migration and to a large extent the cell-division in all but the developing blood-cells. The blood-island mesoderm continues to produce primitive erythroblasts which synthesize haemoglobin, and to form the ill-defined endothelial walls which enclose the blood-cells.

TABLE 1

Washing solution and media

	Washing solution and fluid medium	*Gel medium*
3 per cent. agar solution . . .	—	5
10 per cent. glucose	2	2
Sørensen's PO_4 buffer M/15, pH 4·7 .	2	2
Unbuffered Panett–Compton saline (× 20 concn.)	1	1
Distilled water	15 (14·8)	10 (9·8)
Antimetabolite	— (0·2)	— (0·2)

The washing solution, gel, and fluid media were made up immediately before use from sterile stock solutions in the proportions given in Table 1. Each blastoderm was cultured in a watch-glass containing 5·0 ml. of medium. The antimetabolites 8-azaguanine, fluorophenylalanine, and thienylalanine and their normal analogues guanine and phenylalanine were made up in M NaOH and diluted to make a M/5 stock solution; their concentration when used was equivalent to 10 mg./100 ml. Chloramphenicol was used in fluid media only in a final concentration of 50 mg./100 ml. Crystalline RNAse was dissolved in glucose-PO_4-saline at pH 7·6 to make a stock solution containing 5,000 μg./ml. The final concentration of RNAse in the medium was 250 μg./ml. When an

antimetabolite or the normal analogue was included in the gel medium, the deembryonated blastoderm was washed before its transfer to the gel in a buffered glucose saline solution containing the same amount of the substance concerned as in the gel. RNAse digestion was done in fluid medium, while control blastoderms were suspended in the fluid medium alone for the same period.

The development of haemoglobin was observed by direct microscopy in the living system and histochemically by a method utilizing the peroxidase activity of this protein. Haemoglobin reacts with hydrogen peroxide as substrate and *o*-dianisidine (3, 3′-dimethoxybenzidine) as the H-donor or 'acceptor' to convert the latter into an orange-brown dye by peroxidatic oxidation. Based on the use of *o*-dianisidine by Owen, Silberman, & Got (1958), who used this reagent to demonstrate haemoglobin in zone electrophoresis in a starch gel, the author found it to be an excellent histochemical reagent for haemoglobin identification and estimation (O'Brien, 1960*a*). The following stock solutions may be stored in a refrigerator: (1) *o*-dianisidine solution: 100 mg./70 ml. of ethanol; (2) acetate buffer: 0·15 M at a pH of 4·7; (3) hydrogen peroxide: 100 vol. The staining solution (made up immediately before use) is as follows: *o*-dianisidine solution, 2·0 ml.; acetate buffer, 0·5 ml.; distilled water, 2·0 ml.; hydrogen peroxide, 0·1 ml. This quantity is adequate for one blastoderm. The tissue was transferred directly from the growth medium into the staining solution, stained for 15 minutes, rinsed in distilled water, dehydrated in dioxane, cleared in xylol, and mounted in D.P.X. mounting medium or in Canada balsam.

The degree of haemoglobin development which becomes directly visible in the living blood-islands and the relative amount and intensity of the stain in the cleared and mounted preparation are represented by conventional signs. In the living system:

— no visible colour.
+ isolated occasional pink spots which indicate the early appearance of haemoglobin in groups of cells within blood-islands.
++ numerous but mainly separate pink areas with occasional confluence.
+++ clear red irregular reticulation especially in the peripheral regions extending towards the centre.

In the stained and mounted whole blastoderm:

— no visible reaction (Plate 2, fig. D).
+ a few scattered cells clearly positive and occasional small groups of cells faintly stained (Plate 1, fig. E).
++ reaction more intense. Reticular appearance well defined but pale. Scattered groups of cells deeply stained (Plates 1, fig. F; 2, fig. B).
+++ intense and extensive reaction. Well-defined and deeply stained reticulation. Many deeply stained cells especially in the peripheral regions (Plates 1, fig. C; 2, fig. C).

The difference between the two methods of observation is one of sensitivity. The

o-dianisidine reaction is much more sensitive than direct observation of haemoglobin and has the advantage of being examined in cleared and mounted specimens. Plate 1, figs. C and D, show haemoglobin-positive cells in a typical whole mount photographed through a ×10 and an oil-immersion ×100 objective respectively.

RESULTS

Haemoglobin first became directly visible in the blood-islands of the embryo

TABLE 2

Appearance of haemoglobin in blood-islands of blastoderms de-embryonated at developmental stages 4–10 and transferred to a normal agar gel

Degree of haemoglobin development noted in both the living system and following histochemical localization by the peroxidatic oxidation of *o*-dianisidine

Developmental stage at de-embryonation	*Time on gel*	*No. of embryos used*	*Visible haemoglobin No. of embryos*				*No. of embryos used*	*Haemoglobin peroxidase No. of embryos*			
			—	+	++	+++		—	+	++	+++
	(hrs.)										
4	0	6	6				2	2			
	20	4	4				4	4			
	30	4	3	?			4	2	2		
	48	4	4								
5	0	4	4				2	2			
	12	4	4				4	1	1	2	
	24	4	1	3			4			2	2
	48	4				4	4				4
6	0	8	8				2	2			
	10	8	8				3	1	1	1	
	24	8			2	6	3				3
	36	8				8	1				1
	48	8				8	1				1
7	0	10	10				4	4			
	10	10	8	2			4	2	2		
	20	10	6	3		1	4			1	3
	30	10		2	4	4	2				2
	48	10				10	2				2
8–	0	15	15				4	4			
	5	15	15				4	1	3		
	10	15	6	5	4		4			2	2
	15	15	2	3	10		2				2
	24	15		1	6	8					
	48	15				15					
8	0	19	19				5	3	2		
	5	10	8	2			4	1	2	1	
	10	10	1	3	6		6			2	4
	15	12			4	8	4				4
9	0	12	9	3 ?			6		2	4	
	5	12	3	7	2		3			1	2
	10	9		?	7	1	3				3
	20	6				6	2				2
10	0	5		5			3				3
	5	5			5		3				3
	10	4				4	3				3

between the 8- and 10-somite stage, that is between developmental stages 9 and 10 according to the Hamburger–Hamilton scale (Hamburger & Hamilton, 1951). In the 1- and 2-somite embryos, stages 7 and 8 , haemoglobin was not detectable in the blood-island cells. A general histological stain or blood-stain at this stage shows that the blood-islands are already differentiated into a reticulation of well-defined strongly basophilic regions made up of actively dividing cells.

TABLE 3

Appearance of haemoglobin in blood-islands of blastoderms de-embryonated at developmental stages 4–10

Transferred to gel containing 8-azaguanine in a final concentration of 10 mg./100 ml.

Developmental stage at de-embryonation	*Time on gel*	*No. of embryos used*	*Visible haemoglobin No. of embryos*				*No. of embryos used*	*Haemoglobin peroxidase No. of embryos*			
			—	+	++	+++		—	+	++	+++
	(hrs.)										
4	30	4	4				2	2			
5	24	4	4				2	2			
	48	4	4				2	2			
6	24	5	5				2	2			
	48	7	7				7	7			
7	20	4	4				2	2			
	30	6	6				2	2			
	48	6	6				4	4			
8⁻	10	20	20				6	6			
	24	16	16				4	4			
	30	10	10				4	2	2 ?		
	48	10	10				2	2			
8	10	6	6				4	4			
	24	10	10				10	4	4	2 ?	
	30	10	10				10	5	5		
9	5	9	3	6			5	1	4		
	10	9		2	6	1	5				5
	24	7			1	6	2				2
10	5	6		3	3		2				2
	10	6				6	2				2

Table 2 summarizes the results obtained when different developmental stages were de-embryonated and transferred to a gel medium which contained no inhibitor or antimetabolite, that is a 'normal' gel. The appearance and degree of haemoglobin development both by direct observation in the living system and by histochemical means are related to the developmental time on the gel medium.

Stage 4 blastoderms (stage 4 at the time of de-embryonation and transfer to the gel, i.e. at 0 hours) after 48 hours on the gel did not develop directly visible haemoglobin, although two out of the four gave a weak positive histochemical reaction after 30 hours. All later stages showed clearly a marked haemoglobin synthesis within 24 hours. Stages 5, 6, 7, and 8⁻ all gave a negative reaction at

0 hours. Stage 8 was positive to *o*-dianisidine in two out of five cases at the time of transfer but haemoglobin was just visible in the living system after 5 hours. Haemoglobin was present at 0 hours in all six stage-9 blastoderms tested histochemically but was not directly observed with certainty in the living system until a few hours later. At stage 10 haemoglobin was present in all cases at the time of gel culture.

TABLE 4

Appearance of haemoglobin in blood-islands of de-embryonated blastoderm, developmental stages 4–10

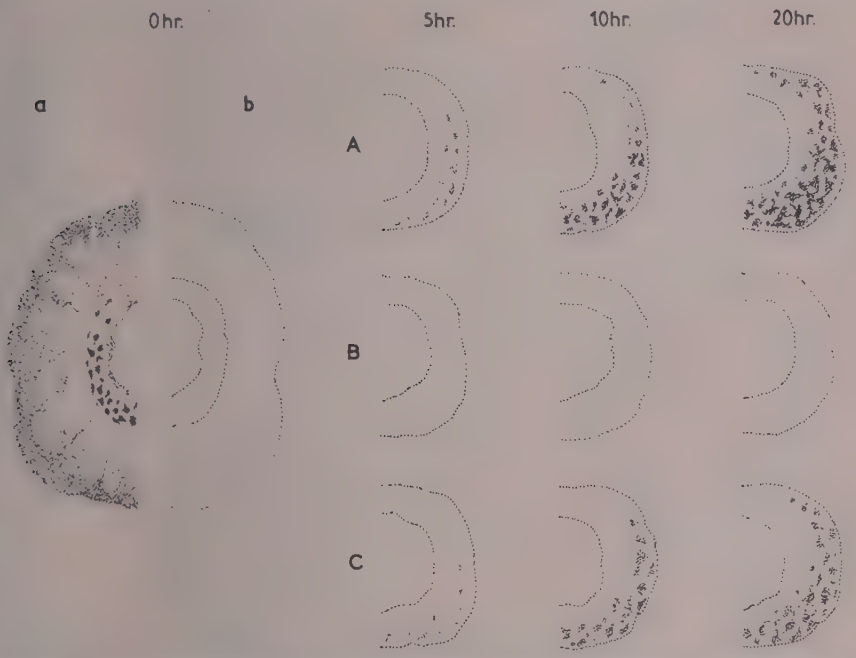
Transferred to gel containing both 8-azaguanine and guanine each in a final concentration of 10 mg./100 ml.

Developmental stage at de-embryonation	*Time on gel*	*No. of embryos used*	*Visible haemoglobin No. of embryos*				*No. of embryos used*	*Haemoglobin peroxidase No. of embryos*			
			—	+	++	+++		—	+	++	+++
	(hrs.)										
4	30	4	4				2	2			
5	24	4	4				2	2			
	48	4	4				2	1	1 ?		
6	24	4	1	3			2			2	
	48	4			4		2				2
7	20	6	6				2		2		
	30	6		4	2		2				2
	48	4		2	2		2				2
8⁻	10	18	15	3 ?			4			3	1
	24	18	6	10	8		4				4
	30	16		3	6	7	2				2
8	10	6	2	4			2			1	1
	24	6	1	1	4		2				2
	30	6			6		2				2
9	5	5		5			2				2
	10	5			3	2	2				2
10	5	5		5			2				2
	10	5			3	2	2				2

These results suggest that up to and including developmental stage 8⁻ the synthetic mechanism for haemoglobin formation had not begun to operate or was not yet complete. Between stage 8⁻ and the latter part of stage 8 the synthesis of haemoglobin has become only just operative in a few scattered centres in the peripheral blood-islands. Stages 9 and beyond showed the onset of effective and active haemoglobin synthesis which is clearly demonstrable at 0 hours, histochemically at stage 9, and visible directly at stage 10.

Table 3 demonstrates the effect upon the appearance of haemoglobin of including the RNA analogue 8-azaguanine in the medium. The results show that there is a developmental stage before which the antimetabolite effectively blocks the formation of haemoglobin but after which it has little effect. The

appearance of haemoglobin in the living system differentiates between stages 8 and 9, while the more sensitive peroxidase reaction shows that in some blastoderms there has been some production of the protein at stage 8. Table 2 indicates that some 50 per cent. of the blastoderms might be expected to show a faint positive reaction at 0 hours, which suggests that stage 8 is a period in which the synthetic mechanism begins haemoglobin production but is also one in which the synthesis cannot be maintained in the presence of 8-azaguanine. Plate 2, fig. D shows the blood-island region of a stage 8⁻ blastoderm which is typical

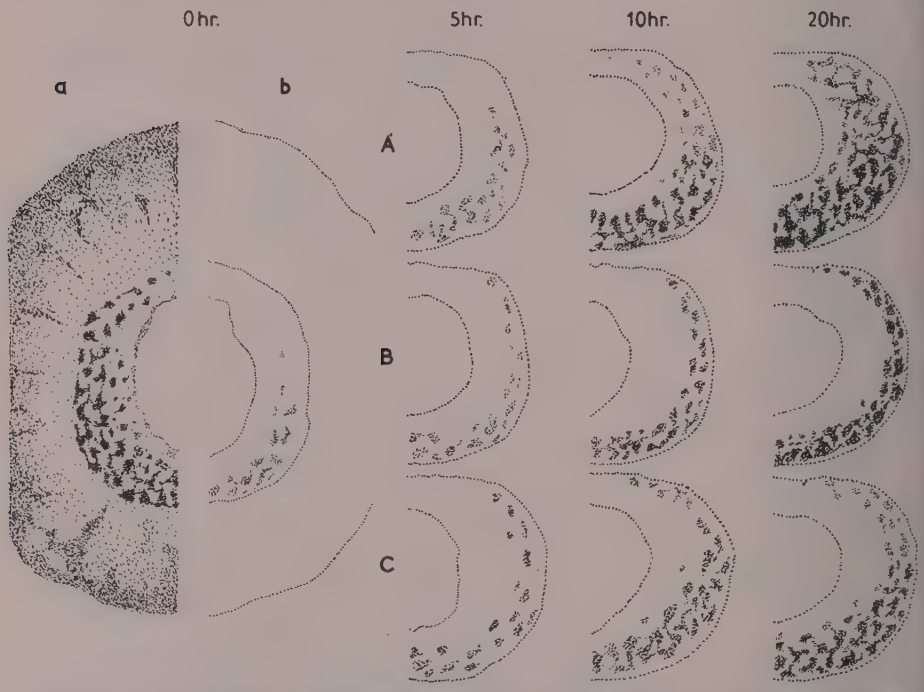


TEXT-FIG. 1. Development of haemoglobin in the blood-islands of a blastoderm de-embryonated at stage 8⁻ (Hamburger & Hamilton) and cultivated on an agar gel. *a*, half-blastoderm at the time of de-embryonation stained by the May Grunwald Giemsa technique to show the extent of blood-island development. *b*, half-blastoderm tested for haemoglobin peroxidase activity. *A*, *B*, and *C* represent the blood vascular regions of de-embryonated blastoderms developed on a normal gel, *A*; a gel containing 8-azaguanine, *B*; a gel containing both azaguanine and guanine, *C*. The development of haemoglobin peroxidase activity is shown for each medium at 5, 10, and 20 hours.

of the azaguanine-treated system after 30 hours, while Plate 2, figs. J and K show almost normal haemoglobin production in peripheral islands by a stage-9 blastoderm in 24 hours on a similar gel. A stage-10 blastoderm, which at 0 hours showed a strongly positive reticulation in both peripheral and central regions, was scarcely distinguishable from the untreated control at 10 hours.

In the series shown in Table 4 the normal base guanine has been added to the medium which already contains 8-azaguanine. Stage 6 produces haemoglobin

but shows some inhibition in that the positive regions were neither as numerous nor as intense. Stage 8⁻ after 24 hours (Plate 2, fig. E) differs from the control (Plate 2, fig. C) in the amount of haemoglobin produced but not in the degree to which the more central islands exhibit synthetic activity. In contrast with stage 8⁻, stage-9 blastoderms show an active haemoglobin synthesis which results at observational level in as much of the protein as the normal system. This active synthesis appeared to some extent restricted to the more peripheral regions of the blastoderm (Plate 2, fig. I).



TEXT-FIG. 2. Development of haemoglobin in a blastoderm de-embryonated at stage 9. Description as for Text-fig. 1.

Tables 3 and 4 demonstrate that the RNA base analogue 8-azaguanine effectively blocked the formation of haemoglobin up to and including stage 8, but not beyond this stage. The addition of an equal amount of the normal base guanine in the presence of its analogue eliminated this inhibition to a considerable extent at stage 6 and above but not at stages 4 and 5.

Text-figs. 1 and 2 summarize this system for two critical stages in the synthesis of haemoglobin. Text-fig. 1 shows a stage 8⁻ de-embryonated blastoderm; (*a*) represents one-half of the blastoderm stained by a general stain at the time of de-embryonation and transfer, to show the development and distribution of the blood-islands in contrast with (*b*) which represents peroxidase-positive regions

at this same stage. *A*, *B*, and *C* show diagrammatically the blood-island region of the blastoderm which has developed on a normal gel, an azaguanine gel, and one containing both azaguanine and guanine, for periods of 5 hours, 10 hours, and 20 hours respectively. *A*, *B*, and *C* all represent the blood-island region stained by the peroxidase reaction.

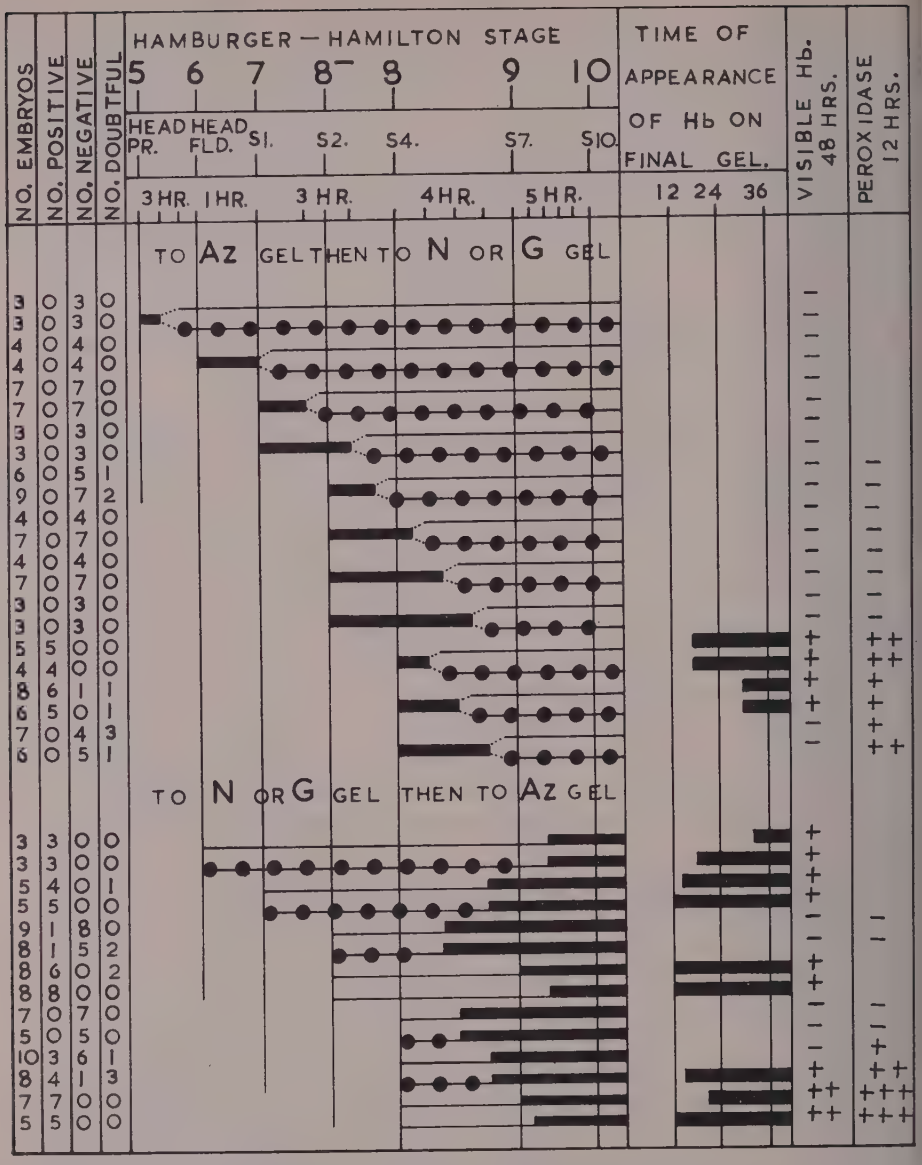
Text-fig. 2 represents the stage-9 system. The essential difference between the two systems is that at stage 8 there is no haemoglobin present at 0 hours or synthesized later when azaguanine is present, while at stage 9 there is some haemoglobin present at 0 hours and its subsequent synthesis is only slightly inhibited by the antimetabolite. In both stages the inhibition by azaguanine does not occur to nearly the same extent when guanine is present at the same concentration.

The following experiments were an attempt to determine: (*a*) whether the inhibition caused by azaguanine occurred when the inhibitor was removed after a short time in contact with the developing blastoderm; (*b*) the extent of that period between stages 8 and 9 when the analogue inhibition first falls off; (*c*) whether the addition of the normal metabolite guanine to the system either before or after contact with azaguanine would modify the inhibition to the same extent as when guanine and azaguanine were present together.

In the first group the de-embryonated blastoderms were incubated on an azaguanine gel for different times at developmental stages 5–8 inclusive. The blastoderms were then washed in several changes of a normal buffered medium at 37·5° C. and then transferred to a normal gel, or to one containing guanine.

In the second group the de-embryonated blastoderms, stages 6–8, were grown for a certain time either on a normal gel or on one containing guanine. They were then washed in medium containing azaguanine and transferred to an azaguanine gel. In both cases the development of haemoglobin was recorded as before.

Text-fig. 3 summarizes this investigation. Azaguanine blocked haemoglobin development up to stage 8 even though the blastoderm was in contact with the inhibitor for only 1 hour. Stage-8 blastoderms formed some haemoglobin when transferred from the azaguanine gel after 1 hour. The appearance of haemoglobin was retarded but became visible in the living system after 2 hours on the azaguanine gel. After 3 hours in contact with the inhibitor there was only a faint peroxidase-positive reaction. Table 2 showed that at stage 9 azaguanine did not block haemoglobin production, so that the period of approximately 4 hours between stages 8 and 9 was the time during which the RNA antimetabolite 8-azaguanine ceased to be an effective block to the synthesis of haemoglobin. The second group of experiments in which the de-embryonated blastoderm was first placed on a normal or a guanine gel and then transferred to an azaguanine gel suggest the following generalization: if the de-embryonated blastoderm, irrespective of the stage at which it was de-embryonated, is allowed to develop on a normal gel for a period equivalent to the time it would have taken as an entire blastoderm to develop to stage 9, then haemoglobin will be



TEXT-FIG. 3. Horizontal rows indicate the types of gel media on which the de-embryonated blastoderms were placed, and the stage of development at which the blastoderm was de-embryonated and transferred from the vitelline membrane to the gel. Thick line represents a gel containing 8-azaguanine (Az gel), thin line represents a normal gel (N gel), and the thin line with solid circles imposed, a gel containing guanine (N+G gel). The developmental stages are from stage 5 to stage 10 inclusive (Hamburger & Hamilton), that is from the head-process (Head Pr.) to the 10-somite stage (S 10). The approximate duration of each stage in hours is given. In the uppermost group of experiments (to Az then to N or N+G gel) the blastoderms were transferred to an Az gel for a time indicated by the length of the thick horizontal line. At the end of this period the blastoderms were retransferred

TABLE 5

De-embryonated blastoderms transferred to a gel medium (Gel 1) after, in some cases, pretreatment in a fluid medium

Retransfer to a second gel (Gel 2) took place in some cases. Developmental time on the media concerned is represented by t, t_1, and t_2. N represents a normal saline or gel medium. Additions to this medium are represented by: RNAse, ribonuclease; ClM, chloramphenicol; PA, phenylalanine; FPA, fluorophenylalanine; Az, 8-azaguanine; G, guanine

No. of embryos used	*Saline medium*	t	*Gel 1*	t_1	*Gel 2*	t_2	*Haemoglobin development*	
							Visible	*Peroxidase*
		(hrs.)		(hrs.)		(hrs.)		
Developmental stage 8⁻								
2	—	—	N+PA	27	—	—	++	
4	—	—	FPA	27	—	—	+	
2	—	—	FPA−PA	27	—	—	++	
2	—	—	FPA	2	N	26	−	
2	—	—	FPA	2	N−PA	26	+	
2	N	1	N	15	—	—	+	++
2	RNAse	1	N	15	—	—	+	+++
2	RNAse	1	N−RNA	15	—	—	+	+++
2	RNAse	1	N	4	Az	15	−	+
2	RNAse	1	N	6	Az	15	−	++
4	ClM	1	N	15	—	—	+	+++
2	ClM−RNAse	1	N	15	—	—	+	+++
2	ClM−RNAse	1	N	6	Az	15	−	++
2	ClM−RNAse	1	FPA	24	—	—	−	++
Developmental stage 9								
1	—	—	FPA	24	—	—	−	
1	—	—	FPA−PA	18	—	—	+++	
1	—	—	FPA	1	N	24	−	
1	—	—	FPA	3	N	24	−	
1	—	—	N	5	FPA	16	++	
2	N	1	N	10	—	—	++	+++
2	RNAse	1	N	10	—	—	++	+++
2	RNAse	1	Az	10	—	—	−	++
2	RNAse	1	Az−G	10	—	—	−	++
2	RNAse	1	FPA	10	—	—	−	++
4	ClM	1	N	10	—	—	++	+++
1	ClM	1	FPA	10	—	—	++	+++
1	ClM−RNAse	1	N	18	—	—	++	+++
1	ClM−RNAse	1	Az	18	—	—	−	+
1	ClM−RNAse	1	Az−G	18	—	—	+	++
1	ClM−RNAse	1	FPA	18	—	—	++	+++

synthesized in the presence of azaguanine. A blastoderm from a normal gel having reached a stage equivalent to stage 8 plus 2 hours (half-way between

either to a N gel or to a N+G gel. The lower group (to N or N+G then to Az gel) was the opposite of the previous treatment. Histograms show the time of appearance of haemoglobin in hours from the time of retransfer to the second gel. The degree of synthesis is represented by negative or positive signs as described in 'Materials and Methods'. Column (Hb 48 hrs.) represents haemoglobin directly visible after 48 hours and column (Perox. 12 hrs.) the intensity of the haemoglobin peroxidase reaction after 12 hours. The columns at the left-hand side of the figure state: the total number of embryos used, the number that showed positive haemoglobin development, the number that were negative, and the number in which there was some doubt whether there was haemoglobin present.

stages 8 and 9) did not in all cases synthesize haemoglobin when transferred to an azaguanine gel but when transferred at stage 8 plus 3 hours synthesis was more probable and at stage 8 plus 4 hours (stage 9) the formation of the protein was almost certain.

The addition of guanine to the gel to which the blastoderm was transferred after a preliminary period in contact with azaguanine (Text-fig. 3, first series) did not modify or relieve the inhibition which had been established while in contact with the inhibitor. The second series (Text-fig. 3) in which the initial incubation was on a normal or a guanine gel did indicate that the addition of guanine tended to increase haemoglobin production when compared with the normal controls. In no case did the presence of guanine in the system result in haemoglobin synthesis when the control from the normal gel was completely inhibited by transfer to an azaguanine gel.

Both sets of experiments suggest that there is a period of only a few hours, between developmental stages 8 and 9, when the effect of the antimetabolite changes from complete inhibition to a degree of inhibition which may well be determined by other factors. The important observation is that, on the one hand, there was no synthesis in the presence of an RNA antimetabolite and, on the other hand, at a developmental stage only a few hours later synthesis takes place even though the antimetabolite is present.

In addition to the more detailed investigation of the RNA antimetabolite 8-azaguanine the effect of some other modifiers of protein synthesis was tested as a preliminary to an extension of this programme. The results obtained so far are not sufficiently extensive to warrant a detailed description. The amino-acid analogues thienylalanine and fluorophenylalanine, a less specific inhibitor of protein synthesis, chloramphenicol, and the effect of ribonuclease treatment were tested for the influence they might have on this synthesis.

Thienylalanine is omitted from Table 5 because it showed no significant effect on haemoglobin synthesis at the concentration used. Fluorophenylalanine caused a considerable and definite inhibition in both stage 8^- and stage 9 blastoderms. Incubation in chloramphenicol for 1 hour prior to transfer to a normal gel resulted in no apparent inhibition when compared with the control blastoderm which was incubated for the same time in a normal medium. The same can be said for pretreatment in RNAse solution. When RNAse treatment was followed by transfer to a gel containing either fluorophenylalanine or azaguanine, then haemoglobin synthesis was inhibited in both stage-8^- and stage-9 blastoderms. That some inhibition occurred in the stage-9 blastoderms indicates that the RNAse has affected the synthetic system, since direct transfer to an azaguanine gel should not restrict haemoglobin formation to the same extent (cf. Table 3). There was no evidence that the presence of chloramphenicol in the RNAse digestion medium had any noticeable effect compared with that found with RNAse alone except in the one case where the blastoderm was transferred to a gel containing fluorophenylalanine. The inhibition produced

by this analogue, independent of RNAse treatment, did not occur when chloramphenicol was present. Why this should be so is not clear; however, it should be emphasized that only one experimental observation was made.

DISCUSSION

The evidence presented suggests that inhibition by the RNA base analogue, 8-azaguanine, is not primarily due to a general inhibition of metabolism, but is more likely to be specifically associated with RNA metabolism. If the production of a ribonucleoprotein template precedes the formation of a protein on that template and if an abnormal RNA is unacceptable to the synthetic mechanism, as the work by Petermann *et al.* (1956) on liver and tumour cells and by Kramer & Straub (1956) with *Staphylococcus aureus* would suggest, then the data described indicate that templates or RNA-associated mechanisms responsible for the production of haemoglobin become functional at or soon after developmental stage 8. This is in close agreement with the finding of D'Amelio & Salvo (1959) who used a serological method combined with agar diffusion. The relative independence of haemoglobin synthesis in the presence of the analogue at stage 9 and after indicates that the mechanism once formed is stable. Creaser (1956) used azaguanine to inhibit the formation of both 'adaptive' and 'constitutive' enzymes in *S. aureus* and obtained results that support this observation. The permanence of azaguanine inhibition as shown by those experiments in which an early-stage blastoderm is removed after 1 hour in contact with the analogue and transferred to a normal gel suggests that the final synthesis is dependent upon RNA-associated structures formed at stage 5.

When the presence of haemoglobin becomes first histochemically detectable in the few scattered erythroblasts among the peripheral blood-islands of the vascular region, most cells show a faint positive reaction in both nucleus and cytoplasm (O'Brien, 1960*b*). Plate 1, fig. E represents a developmental stage only a little later than that referred to above. Some erythroblasts show only a nucleolar or nuclear reaction which though very faint is, nevertheless, positive. It is at this stage of haemoglobin development that azaguanine will still block its production (cf. Table 3, stage 8). Towards the end of stage 8 the majority of cells reacting to peroxidase in the peripheral blood-islands, these being the most developed at any particular stage, show a definite cytoplasmic reaction and in particular a highly reactive perinuclear zone. The antimetabolite did not block the continued synthesis of haemoglobin in those cells showing this reaction pattern.

Text-fig. 4 summarizes the observations made at the intracellular level and relates them to the developmental stage of the blastoderm prior to de-embryonation and transfer to a gel medium. This diagram is in part hypothetical and in part a summary of the experimental data. The innermost ring representing the degree of cell-division is the result of observation rather than a quantitative

estimate of mitotic number and as such is hypothetical. The shaded part gradually decreasing in width from stage 4 to stage 10 plus 10 hours represents the falling off in cell-division within the blood-islands of a de-embryonated blastoderm transferred to a normal gel at stage 4. Nineteen hours later, when the system has reached the stage equivalent in developmental time to a stage-11

TEXT-FIG. 4. Diagrammatic representation of the intracellular appearance of haemoglobin in relation to antimetabolite inhibition and the developmental stage of the de-embryonated blastoderm. For description see text.

blastoderm, cell-division has decreased and during the next 10 hours decreases still further. The next three rings represent the nucleolus, the nucleus, and the cytoplasm, in that order, and the stippling indicates the relative intensity of the haemoglobin peroxidase reaction. The fifth ring states the approximate duration of the developmental stage which is shown in the outermost ring. The times quoted refer to the *in vitro* development of a normal (embryonated) blastoderm. The sixth and seventh rings, which are partly and wholly shaded respectively, represent the degree of inhibition of haemoglobin synthesis by the metabolite analogues 8-azaguanine and fluorophenylalanine respectively.

The pattern of peroxidase activity in the cell which represents the site and relative intensity of haemoglobin synthesis indicates that the nucleolar region is where the mechanism first becomes functional and the first 'template' or 'templates' become operative. Evidence from many sources indicates that protein synthesis is associated with the ribonucleoprotein fraction that appears after fixation as particles of diameter 100 to 150 Å in electron micrographs (Petermann *et al.*, 1954, Palade & Siekevitz, 1956). Similar particles constitute a large proportion of the nucleolar region.

Two points of interest emerge from the data summarized in Text-fig. 4. Firstly, the difference between the inhibition by the RNA base analogue during the early and later stages is suggestive of an increased stability in the template RNA once it has been incorporated into the final synthetic mechanism. Secondly, it is not until cytoplasmic production of the protein has begun that the RNA base analogue ceases to inhibit effectively the synthesis of the protein. If 'template' production is operative rather than a sequential series of reactions in the production of the protein, then the data suggest that the early nucleolar and nuclear sites of synthesis at developmental stages 8^- and 8 are still capable of incorporating abnormal RNA. This implies that the early template retains a higher RNA turnover rate than the templates which appear in the cytoplasm and those which develop later in the nucleolus and nucleus. The intense reaction of the nucleus and nucleolus at stage 10 plus 10 hours supports this hypothesis. It is at the later developmental stages that some erythroblasts show a nuclear reaction which is considerably more intense than that in the cytoplasm. Is this effect related to a falling off in cell-division which might result in an accumulation of haemoglobin within the nucleus which, while division is taking place normally, might pass into the cytoplasm after the dissolution of the nuclear membrane? In the post-mitotic cell or cell whose rate of division has slowed, the passage of protein synthesized in the nucleus into the cytoplasm, is perhaps restricted to the nuclear pores. The very dense and relatively wide perinuclear zone in these cells would support this postulate. Palade (1955) has shown that the mammalian red cell once mature, i.e. anucleate, does not contain cytoplasmic ribonucleoprotein granules in association with the cytoplasmic membranes and the mature cell is believed not to synthesize haemoglobin. The studies by Rabinovitz & Olson (1956) showed that haemoglobin synthesis takes place in association with the ribonucleoprotein of the reticulocyte. In addition, the presence of only a few cells in any cell group at developmental stages 8^- and 8 which show the haemoglobin restricted to the nucleus and a relatively thin perinuclear zone could indicate that these cells have not yet divided since synthesis became detectable. The use of mitotic inhibitors or specific culture techniques which could block cell-division at the various developmental stages would be useful in an investigation of this hypothesis.

The amino-acid analogue fluorophenylalanine is a well-known inhibitor of protein synthesis, and since much of the evidence available suggests that

amino-acids are incorporated into protein as free amino-acids at the site of synthesis, then it might be expected that haemoglobin synthesis would be blocked or inhibited irrespective of the developmental stage the system had reached or the effectiveness of the ribonucleoprotein template. The decrease in the inhibition by fluorophenylalanine when phenylalanine is present also is to be expected from the work of Rabinovitz *et al.* (1954), who demonstrated that fluorophenylalanine is incorporated into a protein only one-twentieth as fast as phenylalanine.

Chloramphenicol, in this system, was not successful as an inhibitor of haemoglobin synthesis by itself. In conjunction with RNAse incubation for 1 hour, followed by transfer to a gel containing azaguanine or fluorophenylalanine, some inhibition of synthesis occurred, but the results are not clear. In the case of azaguanine the outcome did not differ from the control in which chloramphenicol was absent, at both stages 8 and 9. It is not clear why pre-incubation with chloramphenicol and RNAse should show less inhibition than with RNAse alone when both are transferred to a gel containing fluorophenylalanine in particular at developmental stage 9. The data are insufficient to warrant further discussion of this observation from the aspect of mechanism, the more so because the concept that chloramphenicol acts as an amino-acid antimetabolite is probably invalid.

The relatively simple histogenic system used throughout this investigation should be capable of development as an erythroblast culture in a fully augmented fluid medium. This would have an advantage for quantitative chemical estimations, but difficulties would arise with respect to the isolating of the specific stages in the differentiation sequence, as this point is important in an investigation of the 'pre-template' to 'template' step or steps in the synthesis of a protein.

SUMMARY

1. A simple *in vitro* histogenesis is described in which the protein haemoglobin is synthesized in the developing blood-islands of a de-embryonated blastoderm.

2. The spread of the blastoderm which follows de-embryonation when it is cultured on the vitelline membrane according to the New technique is eliminated by transfer to an agar gel medium.

3. Cell-division and differentiation in the blood-islands was not inhibited to the same extent as it was in the other tissues present. The erythroblasts multiply, differentiate, and synthesize haemoglobin.

4. The RNA base analogue and antimetabolite 8-azaguanine effectively blocks the synthesis of haemoglobin up to but not including developmental stage 8 on the Hamburger–Hamilton scale. Stages 9 and 10 synthesize haemoglobin in the presence of this analogue.

5. The inhibition by 8-azaguanine is in part removed when the normal base

guanine is included with it in the medium. The inhibition is not removed to any extent by incubation with guanine either before or after contact with azaguanine.

6. It is concluded that there is a period in developmental stage 8 of only 1 or 2 hours that represents the border line between RNA analogue inhibition and non-inhibition. It is considered that during this period the RNA associated mechanisms or templates for haemoglobin production begin to operate.

7. The amino-acid analogue fluorophenylalanine but not thienylalanine inhibited haemoglobin synthesis.

8. Incubation following de-embryonation in RNAse had little effect on the synthesis. Chloramphenicol proved to be ineffective as an inhibitor of protein synthesis in this system.

9. The data presented are discussed in relation to the sequence in which haemoglobin becomes detectable in the nucleolus, the nucleus, and the cytoplasm of the developing erythroblast.

10. Haemoglobin development is observed directly in the living system and histochemically by the peroxidatic oxidation of *o*-dianisidine.

RÉSUMÉ

1. Une histogénèse *in vitro* simple est décrite, dans laquelle une protéine, l'hémoglobine, est synthétisée par les îlots sanguins en voie de développement dans un blastoderme dont l'embryon a été enlevé.

2. L'extension du blastoderme qui fait suite à l'enlèvement de l'embryon, lorsque la culture se fait sur la membrane vitelline selon la méthode de New, a été éliminée par un transfert sur un milieu à base d'agar.

3. La division cellulaire et la différenciation étaient moins fortement inhibées dans les îlots sanguins que dans les tissus avoisinants. Les érythroblastes se multiplient, se différencient et synthétisent de l'hémoglobine.

4. La 8-azaguanine, qui est un analogue chimique des bases de l'ARN et un antimétabolite, bloque la synthèse de l'hémoglobine jusqu'au stade 8 (non inclus) de la table de Hamburger–Hamilton. Les stades 9 et 10 synthétisent de l'hémoglobine en présence de cet analogue.

5. L'inhibition par la 8-azaguanine est partiellement levée lorsque la base normale (la guanine) est ajoutée au milieu en même temps que l'analogue. Mais l'inhibition n'est nullement levée lorsqu'on ajoute la guanine soit avant, soit après le traitement à l'azaguanine.

6. Il faut en conclure qu'il existe une période de une ou deux heures seulement qui, au stade 8, constitue une frontière entre l'inhibition ou la non-inhibition par l'analogue de l'ARN. On estime que c'est durant cette période que des mécanismes associés à l'ARN ou des modèles (*templates*) pour la production de l'hémoglobine commencent à fonctionner.

7. La fluorophénylalanine (mais pas la thiénylalanine), qui est l'analogue d'un acide aminé, inhibe aussi la synthèse de l'hémoglobine.

8. Un traitement par la ribonucléase, peu après l'enlèvement de l'embryon, n'exerce que peu d'effets sur la synthèse. Le chloramphénicol s'est révélé inefficace en tant qu'inhibiteur de la synthèse protéique dans le système étudié.

9. Les résultats sont discutés en relation avec la séquence selon laquelle l'hémoglobine peut être décelée dans le nucléole, le noyau et le cytoplasme de l'érythroblaste en voie de développement.

10. La formation de l'hémoglobine peut s'observer directement sur le vivant et, cytochimiquement, par l'oxydation peroxydasique de la *o*-dianisidine.

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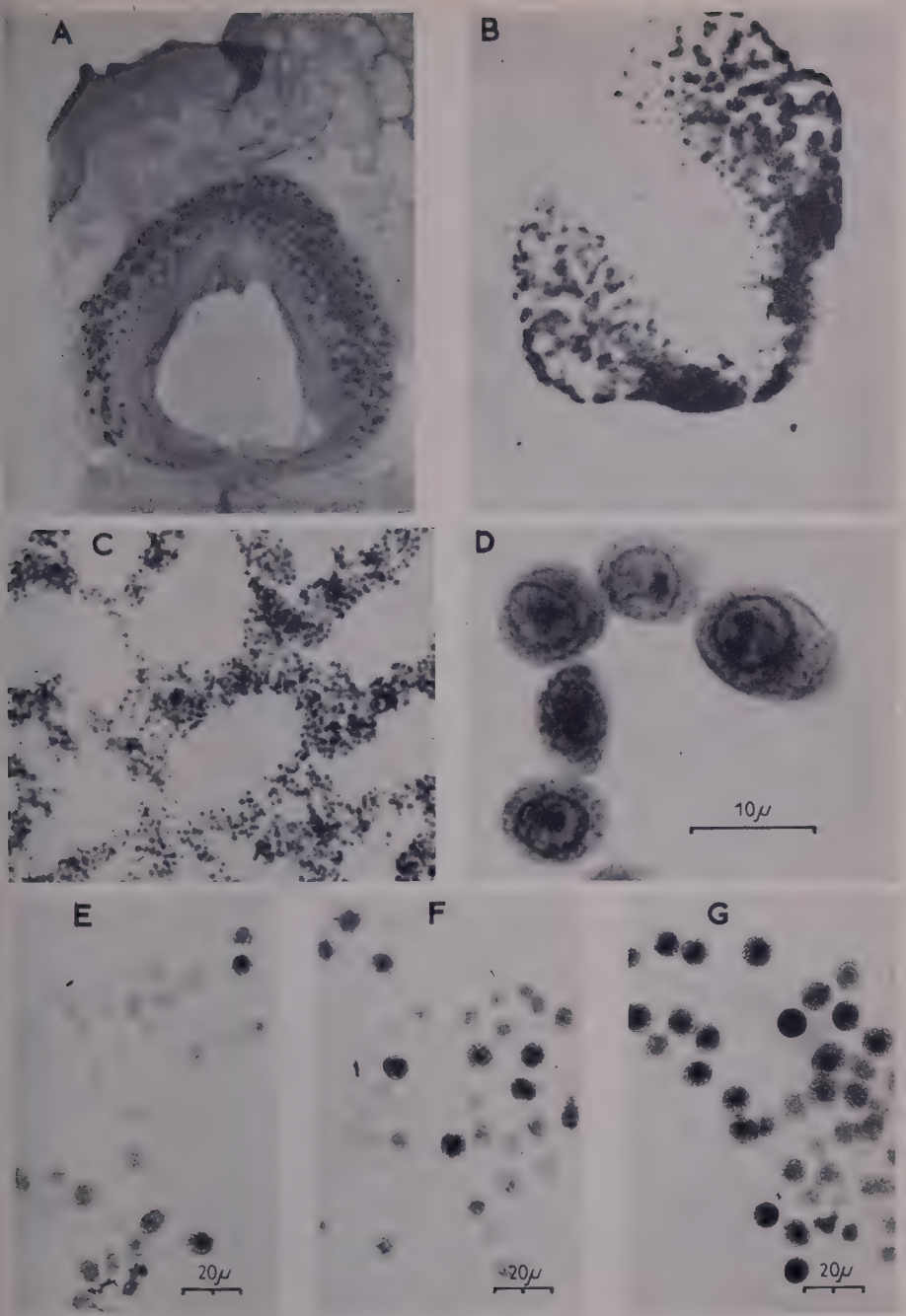

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Plate 1

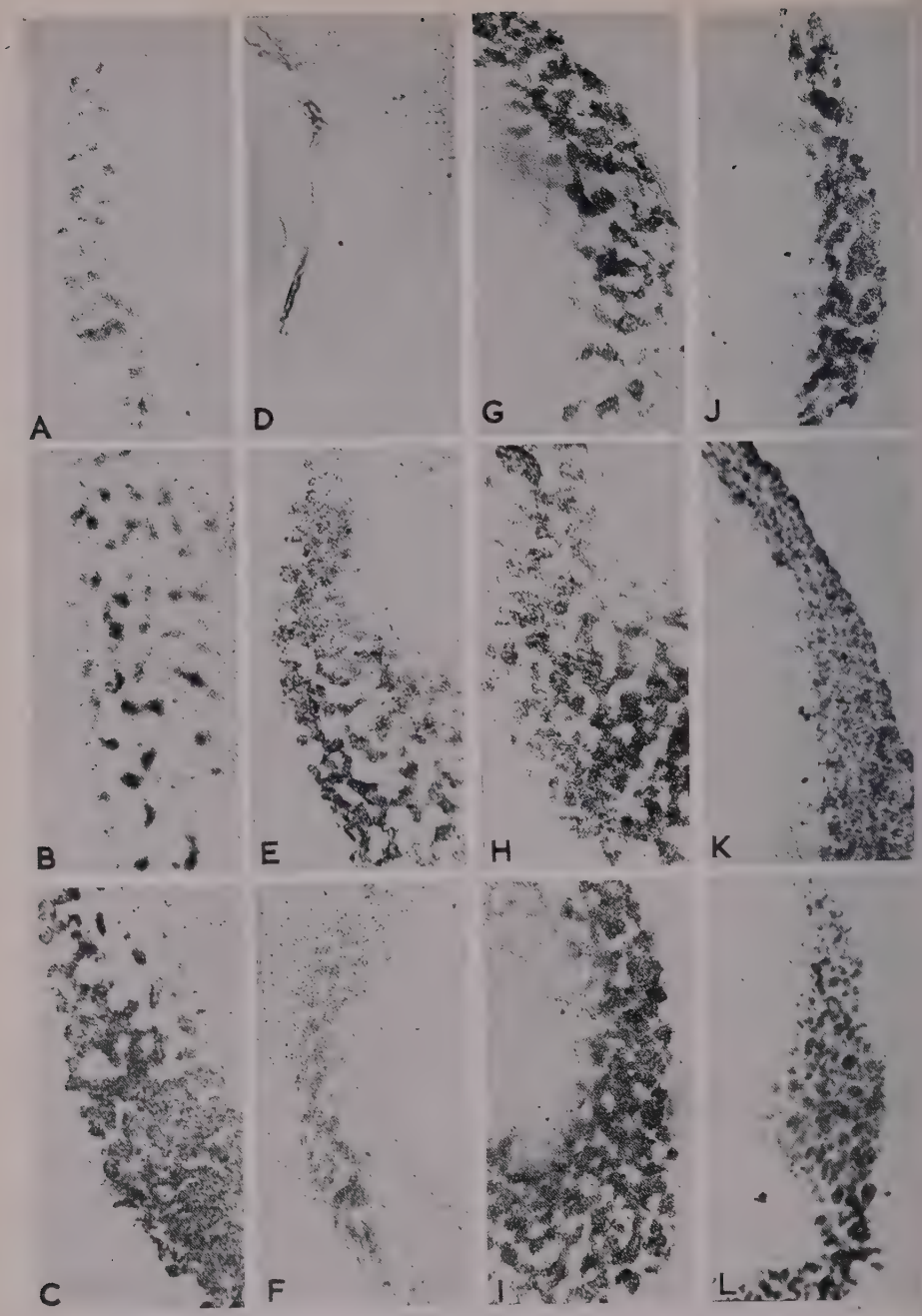

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Plate 2

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EXPLANATION OF PLATES

PLATE 1

FIG. A. De-embryonated, stage-9 blastoderm cultured for 24 hours on a buffered glucose-saline agar gel. Stained in *o*-dianisidine and counterstained in light green. Blood-islands intensely positive for haemoglobin, other tissus clearly differentiated. Cleared and mounted whole in D.P.X. mounting medium. ×10.

FIG. B. De-embryonated blastoderm as in fig. A, but not counterstained, demonstrating the specificity of the reaction and the absence of background stain. ×15.

FIG. C. Reticulation of blood-island tissue photographed in a whole mount. Peroxidase reaction confined to the developing erythroblasts. Ectoderm, non-vascular mesoderm, and entoderm unstained.

FIG. D. Erythroblasts containing haemoglobin stained in *o*-dianisidine. Nucleolus and perinuclear zone showing intense reaction.

FIG. E. Smear of erythroblasts from the blood-islands of a stage-9 blastoderm. The presence of haemoglobin only just detectable in the majority of cells.

FIG. F. Smear of erythroblasts from blood-islands of a later developmental stage.

FIG. G. Smear of erythroblasts from a stage-10 blastoderm. At this stage haemoglobin is visible in the living tissue, and the peroxidase reaction intense in many cells.

PLATE 2

The figures show a portion of the developing blood-island region in de-embryonated blastoderms which have been cultured on either a normal gel, a gel containing azaguanine, or a gel containing both azaguanine and guanine. The blastoderms have been stained in *o*-dianisidine, cleared, and mounted whole. All were photographed under the same conditions and enlarged to the same extent. ×30.

FIG. A. Stage 8⁻ blastoderm de-embryonated and cultured on a normal gel for 6 hours.

FIG. B. Stage 8⁻ blastoderm de-embryonated and cultured on a normal gel for 10 hours.

FIG. C. Stage 8⁻ blastoderm de-embryonated and cultured on a normal gel for 24 hours.

FIG. D. Stage 8⁻ blastoderm de-embryonated and cultured on a gel containing 8-azaguanine for 30 hours.

FIG. E. Stage 8⁻ blastoderm de-embryonated and cultured on a gel containing both guanine and azaguanine for 24 hours.

FIG. F. Stage 9 blastoderm at time of transfer to gel.

FIG. G. Stage 9 blastoderm de-embryonated and cultured on a normal gel for 5 hours.

FIG. H. Stage 9 blastoderm de-embryonated and cultured on a normal gel for 10 hours.

FIG. I. Stage 9 blastoderm de-embryonated and cultured on a normal gel for 20 hours.

FIG. J. Stage 9 de-embryonated blastoderm after 10 hours on a gel containing azaguanine.

FIG. K. Stage 9 de-embryonated blastoderm after 24 hours on a gel containing azaguanine.

FIG. L. Stage 9 de-embryonated blastoderm after 24 hours on a gel containing both guanine and azaguanine.

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